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(54) Title: AN IMPROVED LAUNDRY DETERGENT COMPOSITION COMPRISING AMYLASE

(57) Abstract

Laundry detergents comprising novel alpha-amylase mutants derived from the DNA sequences of naturally occurring or recombinant alpha-amylases are disclosed. The mutant alpha-amylases, in general, are obtained by *in vitro* modifications of a precursor DNA sequence encoding the naturally occurring or recombinant alpha-amylase to generate the substitution (replacement) or deletion of one or more oxidizable amino acid residues in the amino acid sequence of a precursor alpha-amylase. Such laundry detergents containing mutant alpha-amylases have increased cleaning ability stability and/or altered pH performance profiles and/or altered thermal stability as compared to the precursor.

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AN IMPROVED LAUNDRY DETERGENT COMPOSITION COMPRISING AMYLASE

Field of the Invention

The present invention relates to laundry detergents containing novel alpha-amylase mutants having an amino acid sequence not found in nature, such alpha-amylase mutants having an amino acid sequence, wherein one or more amino acid residue(s) of a precursor alpha-amylase, specifically any oxidizable amino acid, have been substituted with a different amino acid. The mutant enzymes of the laundry detergents of the present invention exhibit altered stability/activity profiles including but not limited to altered oxidative stability, altered pH performance profile, altered specific activity and/or altered thermostability.

Background of the Invention

Alpha-amylases (alpha-1,4-glucan-4-glucanohydrolase, EC3.2.1.1) hydrolyze internal alpha-1,4-glucosidic linkages in starch largely at random, to produce smaller molecular weight malto-dextrins. Alpha-amylases are of considerable commercial value, being used in the initial stages (liquefaction) of starch processing; in alcohol production; as cleaning agents in detergent matrices; and in the textile industry for starch desizing. Alpha-amylases are produced by a wide variety of microorganisms including *Bacillus* and *Aspergillus*, with most commercial amylases being produced from bacterial sources such as *B. licheniformis*, *B. amyloliquefaciens*, *B. subtilis*, or *B. stearothermophilus*. In recent years the preferred enzymes in commercial use have been those from *B. licheniformis* because of their heat stability and performance, at least at neutral and mildly alkaline pH's.

Previously there have been studies using recombinant DNA techniques to explore which residues are important for the catalytic activity of amylases and/or to explore the effect of modifying certain amino acids within the active site of various amylases (Vihinen, M. et

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al. (1990) *J. Bichem.* **107**:267-272; Holm, L. et al. (1990) *Protein Engineering* **3**:181-191; Takase, K. et al. (1992) *Biochimica et Biophysica Acta*, **1120**:281-288; Matsui, I. et al. (1992) *Febs Letters* Vol. 310, No. 3, pp. 216-218); which residues are important for thermal stability (Suzuki, Y. et al. (1989) *J. Biol. Chem.* **264**:18933-18938); and one group has used such methods to introduce mutations at various histidine residues in a *B. licheniformis* amylase, the rationale for making substitutions at histidine residues was that *B. licheniformis* amylase (known to be thermostable) when compared to other similar *Bacillus* amylases, has an excess of histidines and, therefore, it was suggested that replacing a histidine could affect the thermostability of the enzyme (Declerck, N. et al. (1990) *J. Biol. Chem.* **265**:15481-15488; FR 2 665 178-A1; Joyet, P. et al. (1992) *Bio/Technology* **10**:157 1583).

It has been found that alpha-amylase is inactivated by hydrogen peroxide and other oxidants at pH's between 4 and 10.5 as described in the examples herein. Commercially, alpha-amylase enzymes can be used under dramatically different conditions such as both high and low pH conditions, depending on the commercial application. For example, alpha-amylases may be used in the liquefaction of starch, a process preferably performed at a low pH (pH <5.5). On the other hand, amylases may be used in commercial dish care or laundry detergents, which often contain oxidants such as bleach or peracids, and which are used in much more alkaline conditions.

In order to alter the stability or activity profile of amylase enzymes under varying conditions, [it has been found that selective] replacement, substitution or deletion of oxidizable amino acids, such as a methionine, tryptophan, tyrosine, histidine or [cysteine], results in an altered profile of the variant enzyme as compared to its precursor. Because currently commercially available amylases are not acceptable (stable) under various conditions, there is a need for an amylase having an altered stability and/or activity profile. [This alt red stability (oxidative, thermal or pH)]

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performance profile) can be achieved while maintaining adequate enzymatic activity, as compared to the wild-type or precursor enzyme. The characteristic affected by introducing such mutations may be a change in oxidative stability while maintaining thermal stability or vice versa. Additionally, the substitution of different amino acids for an oxidizable amino acid in the alpha-amylase precursor sequence or the deletion of one or more oxidizable amino acid(s) may result in altered enzymatic activity at a pH other than that which is considered optimal for the precursor alpha-amylase. In other words, the mutant enzymes of the present invention may also have altered pH performance profiles, which may be due to the enhanced oxidative stability of the enzyme.

#### Summary of the Invention

The present invention relates to novel laundry detergent compositions comprising alpha-amylase mutants that are the expression product of a mutated DNA sequence encoding an alpha-amylase, the mutated DNA sequence being derived from a precursor alpha-amylase by the deletion or substitution (replacement) of one or more oxidizable amino acid. In one preferred embodiment of the present invention the mutant results from substituting a different amino acid for one or more methionine residue(s) in the precursor alpha-amylase. In another embodiment of the present invention the mutants comprise substitution of one or more tryptophan residue alone or in combination with the substitution of one or more methionine residue in the precursor alpha-amylase. Such mutant alpha-amylases, in general, are obtained by *in vitro* modification of a precursor DNA sequence encoding a naturally occurring or recombinant alpha-amylase to encode the substitution or deletion of one or more amino acid residues in a precursor amino acid sequence.

Preferably the substitution or deletion of one or more amino acid in the amino acid sequence is due to the replacement or deletion of one or more methionine, tryptophan, cysteine, histidine or tyrosine residues in such sequence, most preferably the residue which is

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changed is a methionine residue. The oxidizable amino acid residues may be replaced by any of the other 20 naturally occurring amino acids. If the desired effect is to alter the oxidative stability of the precursor, the amino acid residue may be substituted with a non-oxidizable amino acid (such as alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, or valine) or another oxidizable amino acid (such as cysteine, methionine, tryptophan, tyrosine or histidine, listed in order of most easily oxidizable to less readily oxidizable). Likewise, if the desired effect is to alter thermostability, any of the other 20 naturally occurring amino acids may be substituted (i.e., cysteine may be substituted for methionine).

Preferred laundry detergents comprise mutants comprising the substitution of a methionine residue equivalent to any of the methionine residues found in *B. licheniformis* alpha-amylase (+8, +15, +197, +256, +304, +366 and +438). Most preferably the methionine to be replaced is a methionine at a position equivalent to position +197 or +15 in *B. licheniformis* alpha-amylase. Preferred substitute amino acids to replace the methionine at position +197 are alanine (A), isoleucine (I), threonine (T) or cysteine (C). The preferred substitute amino acids at position +15 are leucine (L), threonine (T), asparagine (N), aspartate (D), serine (S), valine (V) and isoleucine (I), although other substitute amino acids not specified above may be useful. Two specifically preferred mutants of the present invention are M197T and M15L.

Another embodiment of this invention relates to laundry detergents comprising mutants comprising the substitution of a tryptophan

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residue equivalent to any of the tryptophan residues found in *B. licheniformis* alpha-amylase (see Fig. 2). Preferably the tryptophan to be replaced is at a position equivalent to +138 in *B. licheniformis* alpha-amylase. A mutation (substitution) at a tryptophan residue may be made alone or in combination with mutations at other oxidizable amino acid residues. Specifically, it may be advantageous to modify by substitution at least one tryptophan in combination with at least one methionine (for example, the double mutant +138/+197).

The alpha-amylase mutants included in the laundry detergents of the present invention, in general, exhibit altered oxidative stability in the presence of hydrogen peroxide and other oxidants such as bleach or peracids, or, more specifically, milder oxidants such as chloramine-T. Mutant enzymes having enhanced oxidative stability will be useful in extending the shelf life and bleach, perborate, percarbonate or peracid compatibility of amylases used in cleaning products. Accordingly, a preferred embodiment of the present invention comprises a laundry detergent comprising the mutant alpha-amylases of the invention and further comprising a bleach or peracid compound. A particularly preferred embodiment of the invention is a laundry detergent comprising the mutant alpha-amylases according to the invention which has a pH above about 10 and more preferably of between about 10 and about 12. Also preferred is a granular laundry detergent having a pH of between about 10 and about 12 and further containing a bleach or peracid compound.

Mutant enzymes according to the invention are also surprisingly characterized by having superior activity in the neutral pH ranges when compared to wild type or non-inventive amylases. Accordingly, another particularly preferred embodiment comprises a laundry detergent comprising the mutant alpha-amylases of the invention and having a pH of between about 5.0 and about 10.0, more preferably between 6.0 and about 10.0. A most preferred embodiment is a liquid laundry detergent having a pH between about 6.0 and about 10.0.

Similarly, reduced oxidative stability may be useful in industrial processes that require the rapid and efficient quenching of enzymatic activity. The mutant enzymes of the present invention may also demonstrate a broadened pH performance profile whereby mutants such as M15L show stability for low pH starch liquefaction and mutants such as M197T show stability at high pH cleaning product conditions. The mutants of the present invention may also have altered thermal stability whereby the mutant may have enhanced stability at either high or low temperatures. It is understood that any change (increase or decrease) in the mutant's enzymatic characteristic(s), as compared to its precursor, may be beneficial depending on the desired purpose and formulation of the laundry detergent comprising the mutant alpha-amylase.

The preferred laundry detergents of the invention comprise alpha-amylase mutants derived from a *Bacillus* strain such as *B. licheniformis*, *B. amyloliquefaciens*, and *B. stearothermophilus*, and most preferably from *Bacillus licheniformis*.

In another aspect of the present invention there is provided a laundry detergent comprising a novel form of the alpha-amylase normally produced by *B. licheniformis*. This novel form, designated as the A4 form, has an additional four alanine residues at the N-terminus of the secreted amylase. (Fig. 4b.) Derivatives or mutants of the A4 form of alpha-amylase are encompassed within the present invention. By derivatives or mutants of the A4 form, it is meant that the present invention comprises the A4 form alpha-amylase containing one or more additional mutations such as, for example, mutation (substitution, replacement or deletion) of one or more oxidizable amino acid(s).

A composition embodiment of the present invention comprises laundry detergent compositions, liquid, gel or granular, comprising the alpha-amylase mutants described herein. Preferred are detergent compositions comprising a +197 position mutant either alone or in combination with other enzymes such as endoglycosidases,

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cellulases, proteases, lipases or other amylase enzymes. Particularly preferred are laundry detergent compositions comprising a M15X/W138X/M197X mutant, and most preferably a M15T/W138Y/M197T ("TYT") mutant. Additionally, it is contemplated that the compositions of the present invention may include an alpha-amylase mutant having more than one site-specific mutation.

According to a process embodiment of the present invention, the laundry detergent composition of the present invention is used in a method to clean soiled laundry.

**Brief Description of the Drawings**

Fig. 1a-1c shows the DNA sequence of the gene for alpha-amylase from *B. licheniformis* (NCIB8061), Seq ID No 31, and deduced translation product as described in Gray, G. et al. (1986) J. Bacter. 166:635-643.

Fig. 2 shows the amino acid sequence of the mature alpha-amylase enzyme from *B. licheniformis* (NCIB8061), Seq ID No 32.

Fig. 3a-3b shows an alignment of primary structures of *Bacillus* alpha-amylases. The *B. licheniformis* amylase (Am-Lich), Seq ID No 33, is described by Gray, G. et al. (1986) J. Bact. 166:635-643; the *B. amyloliquefaciens* amylase (Am-Amylo), Seq ID No 34, is described by Takkinen, K. et al. (1983) J. Biol. Chem. 258:1007-1013; and the *B. stearothermophilus* (Am-Stearo), Seq ID No 35, is described by Ihara, H. et al. (1985) J. Biochem. 98:95-103.

Fig. 4a shows the amino acid sequence of the mature alpha-amylase variant M197T, Seq ID No 36.

Fig. 4b shows the amino acid sequence of the A4 form of alpha-amylase from *B. licheniformis* NCIB8061, Seq ID No 37. Numbering is from the N-terminus, starting with the four additional alanines.

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Fig. 5 shows plasmid pA4BL wherein BLAA refers to *B. licheniformis* alpha-amylase gene, *PstI* to *SstI*; *Amp<sup>R</sup>* refers to the ampicillin-resistant gene from pBR322; and *CAT* refers to the Chloramphenicol-resistant gene from pC194.

Fig. 6 shows the signal sequence-mature protein junctions for *B. licheniformis* (Seq ID No 38), *B. subtilis* (Seq ID No 39), *B. licheniformis* in pA4BL (Seq ID No 40) and *B. licheniformis* in pBLapr (Seq ID No 41).

Fig. 7a shows inactivation of certain alpha-amylases (Spezyme® AA20 and M197L (A4 form) with 0.88M H<sub>2</sub>O<sub>2</sub> at pH 5.0, 25°C.

Fig. 7b shows inactivation of certain alpha-amylases (Spezyme® AA20, M197T) with 0.88M H<sub>2</sub>O<sub>2</sub> at pH 10.0, 25°C.

Fig. 7c shows inactivation of certain alpha-amylases (Spezyme® AA20, M15L) with 0.88M H<sub>2</sub>O<sub>2</sub> at pH 5.0, 25°C.

Fig. 8 shows a schematic for the production of M197X cassette mutants.

Fig. 9 shows expression of M197X variants.

Fig. 10 shows thermal stability of M197X variants at pH 5.0, 5mM CaCl<sub>2</sub> at 95°C for 5 mins.

Figs. 11a and 11b show inactivation of certain amylases in automatic dish care detergents. Fig. 11a shows the stability of certain amylases in Cascade™ (a commercially available dish care product) at 65°C in the presence or absence of starch. Fig. 11b shows the stability of certain amylases in Sunlight™ (a commercially available dish care product) at 65°C in the presence or absence of starch.

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Fig. 12 shows a schematic for the production of M15X cassette mutants.

Fig. 13 shows expression of M15X variants.

Fig. 14 shows specific activity of M15X variants on soluble starch.

Fig. 15 shows heat stability of M15X variants at 90°C, pH 5.0, 5mM CaCl<sub>2</sub>, 5 mins.

Fig. 16 shows specific activity on starch and soluble substrate, and performance in jet liquefaction at pH 5.5, of M15 variants as a function of percent activity of *B. licheniformis* wild-type.

Fig. 17 shows the inactivation of *B. licheniformis* alpha-amylase (AA20 at 0.65 mg/ml) with chloramine-T at pH 8.0 as compared to variants M197A (1.7 mg/ml) and M197L (1.7 mg/ml).

Fig. 18 shows the inactivation of *B. licheniformis* alpha-amylase (AA20 at 0.22 mg/ml) with chloramine-T at pH 4.0 as compared to variants M197A (4.3 mg/ml) and M197L (0.53 mg/ml).

Fig. 19 shows the reaction of *B. licheniformis* alpha-amylase (AA20 at 0.75 mg/ml) with chloramine-T at pH 5.0 as compared to double variants M197T/W138F (0.64 mg/ml) and M197T/W138Y (0.60 mg/ml).

Fig. 20 shows the stability testing results of various alpha-amylase multiple mutants incorporated in automatic dish detergent (ADD) formulations at temperatures from room temperature increased to 65°C.

Fig. 21 shows the stability of certain amylase mutants (compared to wild-type) in an automatic dish detergent at room temperature over 0-30 days, as determined by percent activity remaining over time.

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Fig. 22 shows the stability of certain amylase mutants (compared to wild-type) in an automatic dish detergent at 38°C (100°F) with 80% relative humidity over 0-30 days.

Fig. 23 shows the pH activity profile of certain amylases on a Phadebas substrate at 25°C at neutral and alkaline pH.

Fig. 24 shows the stability of certain amylases to peracetic acid over time at pH 9.3 and 52°C.

Fig. 25 shows the relative cleaning ability of amylase according to the invention ("TYT") compared to Termamyl amylase in liquid laundry detergent at 40°C in terms of reflectance (delta from control) vs. ppm amylase added.

Fig. 26 shows the relative cleaning ability of amylase according to the invention ("TYT") compared to Termamyl amylase in liquid laundry detergent at 55°C in terms of reflectance (delta from control) vs. ppm amylase added.

Fig. 27 shows the wash performance of amylase according to the invention in commercially available detergent in terms of reflectance (delta from control) vs. ppm amylase added.

#### Detailed Description of the Invention

It is believed that amylases used in starch liquefaction may be subject to some form of inactivation due to some activity present in the starch slurry (see commonly owned US applications 07/785,624 and 07/785,623 and US Patent 5,180,669, issued January 19, 1993, incorporated herein by reference). Furthermore, use of an amylase in the presence of oxidants, such as in bleach- or peracid-containing detergents, may result in partial or complete inactivation of the amylase. Therefore, the present invention focuses on altering the oxidative sensitivity of amylases which are added to laundry detergents. The mutant enzymes in the laundry detergents of the present invention may also have an altered pH

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profile and/or altered thermal stability which may be due to the enhanced oxidative stability of the enzyme at low or high pH's.

Alpha-amylase as used herein includes naturally occurring amylases as well as recombinant amylases. Preferred amylases in the present invention are alpha-amylases derived from *B. licheniformis* or *B. stearothermophilus*, including the A4 form of alpha-amylase derived from *B. licheniformis* as described herein, as well as fungal alpha-amylases such as those derived from *Aspergillus* (i.e., *A. oryzae* and *A. niger*).

Recombinant alpha-amylases refers to an alpha-amylase in which the DNA sequence encoding the naturally occurring alpha-amylase is modified to produce a mutant DNA sequence which encodes the substitution, insertion or deletion of one or more amino acids in the alpha-amylase sequence. Suitable modification methods are disclosed herein, and also in commonly owned US Patents 4,760,025 and 5,185,258, the disclosure of which are incorporated herein by reference.

Homologies have been found between almost all endo-amylases sequenced to date, ranging from plants, mammals, and bacteria (Nakajima, R.T. et al. (1986) *Appl. Microbiol. Biotechnol.* 23:355-360; Rogers, J.C. (1985) *Biochem. Biophys. Res. Commun.* 128:470-476). There are four areas of particularly high homology in certain *Bacillus* amylases, as shown in Fig. 3, wherein the underlined sections designate the areas of high homology. Further, sequence alignments have been used to map the relationship between *Bacillus* endo-amylases (Feng, D.F. and Doolittle, R.F. (1987) *J. Molec. Evol.* 35:351-360). The relative sequence homology between *B. stearothermophilus* and *B. licheniformis* amylase is about 66%, as determined by Holm, L. et al. (1990) *Protein Engineering* 3 (3) pp. 181-191. The sequence homology between *B. licheniformis* and *B. amyloliquefaciens* amylases is about 81%, as per Holm, L. et al., *supra*. While sequence homology is important, it is generally recognized that structural homology is also important in comparing

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amylases or other enzymes. For example, structural homology between fungal amylases and bacterial (*Bacillus*) amylase have been suggested and, therefore, fungal amylases are encompassed within the present invention.

An alpha-amylase mutant has an amino acid sequence which is derived from the amino acid sequence of a precursor alpha-amylase. The precursor alpha-amylases include naturally occurring alpha-amylases and recombinant alpha-amylases (as defined). The amino acid sequence of the alpha-amylase mutant is derived from the precursor alpha-amylase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the precursor DNA sequence which encodes the amino acid sequence of the precursor alpha-amylase rather than manipulation of the precursor alpha-amylase enzyme per se. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein and in commonly owned US patent 4,760,025 and 5,185,258.

Specific residues corresponding to positions M197, M15 and W138 of *Bacillus licheniformis* alpha-amylase are identified herein for substitution or deletion, as are all methionine, histidine, tryptophan, cysteine and tyrosine positions. The amino acid position number (i.e., +197) refers to the number assigned to the mature *Bacillus licheniformis* alpha-amylase sequence presented in Fig. 2. The invention, however, is not limited to the mutation of this particular mature alpha-amylase (*B. licheniformis*) but extends to precursor alpha-amylases containing amino acid residues at positions which are equivalent to the particular identified residue in *B. licheniformis* alpha-amylase. A residue (amino acid) of a precursor alpha-amylase is equivalent to a residue of *B. licheniformis* alpha-amylase if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to a specific residue or portion of that residue in *B. licheniformis* alpha-amylase (i.e., having the same or similar

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functional capacity to combine, react, or interact chemically or structurally).

In order to establish homology to primary structure, the amino acid sequence of a precursor alpha-amylase is directly compared to the *B. licheniformis* alpha-amylase primary sequence and particularly to a set of residues known to be invariant to all alpha-amylases for which sequence is known, as seen in Fig. 3. It is possible also to determine equivalent residues by tertiary structure: crystal structures have been reported for porcine pancreatic alpha-amylase (Buisson, G. et al. (1987) EMBO J. 6:3909-3916); Taka-amylase A from *Aspergillus oryzae* (Matsuura, Y. et al. (1984) J. Biochem. (Tokyo) 95:697-702); and an acid alpha-amylase from *A. niger* (Boel, E. et al. (1990) Biochemistry 29:6244-6249), with the former two structures being similar. There are no published structures for *Bacillus* alpha-amylases, although there are predicted to be common super-secondary structures between glucanases (MacGregor, E.A. & Svensson, B. (1989) Biochem. J. 259:145-152) and a structure for the *B. stearothermophilus* enzyme has been modeled on that of Taka-amylase A (Holm, L. et al. (1990) Protein Engineering 3:181-191). The four highly conserved regions shown in Fig. 3 contain many residues thought to be part of the active-site (Matsuura, Y. et al. (1984) J. Biochem. (Tokyo) 95:697-702; Buisson, G. et al. (1987) EMBO J. 6:3909-3916; Vihinen, M. et al. (1990) J. Biochem. 107:267-272) including, in the *licheniformis* numbering, His105; Arg229; Asp231; His235; Glu261 and Asp328.

Expression vector as used herein refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome-binding sites, and sequences which control termination of transcription and translation. A preferred promoter is the *B. subtilis* *aprE* promoter. The vector may be a plasmid, a phage particle, or simply

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a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, plasmid and vector are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include amylases produced by other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

Host strains (or cells) useful in the present invention generally are procaryotic or eucaryotic hosts and include any transformable microorganism in which the expression of alpha-amylase can be achieved. Specifically, host strains of the same species or genus from which the alpha-amylase is derived are suitable, such as a *Bacillus* strain. Preferably an alpha-amylase negative *Bacillus* strain (genes deleted) and/or an alpha-amylase and protease deleted *Bacillus* strain such as *Bacillus subtilis* strain BG2473 ( $\Delta$ amyE,  $\Delta$ aprE,  $\Delta$ npr) is used. Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the alpha-amylase and its variants (mutants) or expressing the desired alpha-amylase.

Preferably the mutants of the present invention are secreted into the culture medium during fermentation. Any suitable signal sequence, such as the aprE signal peptide, can be used to achieve secretion.

Many of the alpha-amylase mutants of the present invention are useful in formulating various detergent compositions, particularly certain laundry detergent cleaning compositions, and especially those cleaning compositions containing known oxidants, such as bleach or peracid compounds. Alpha-amylase mutants of the invention can be formulated into known powdered, liquid or gel detergents having pH between about 4.5 to about 12.0, preferably

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between about 5.0 and about 10.0 and most preferably between about 6.0 and about 10.0. An additional preferred embodiment comprises laundry detergents having a pH of between about 10.0 and about 12.0, wherein bleach is present in the composition. Suitable granular amylase containing compositions may be made as described in commonly owned US patent applications 07/429,881, 07/533,721 and 07/957,973, all of which are incorporated herein by reference. These detergent cleaning compositions can also contain other enzymes, such as known proteases, lipases, cellulases, endoglycosidases or other amylases, as well as builders, stabilizers or other excipients known to those skilled in the art. These enzymes can be present as co-granules or as blended mixes or in any other manner known to those skilled in the art.

Furthermore, it is contemplated by the present invention that multiple mutants may be useful in cleaning or other applications. For example, a mutant enzyme having changes at both +15 and +197 may exhibit enhanced performance useful in a cleaning product or a multiple mutant comprising changes at +197 and +138 may have improved performance. Specifically preferred mutant enzymes for use in detergent products, and particularly laundry detergent formulations, include but are not limited to M15T/M197T; M15S/M197T; W138Y/M197T; M15S/W138Y/M197T; and M15T/W138Y/M197T ("TYT").

Another embodiment of the present invention comprises the combination of the mutant alpha-amylase enzymes described herein in combination with other enzymes (i.e., proteases, lipases, cellulases, etc.), and preferably oxidatively stable proteases. Suitable oxidatively stable proteases include genetically engineered proteases such as those described in US Re 34,606, incorporated herein by reference, as well as commercially available enzymes such as DURAZYM (Novo Nordisk), MAXAPEM (Gist-brocades) and PURAFECT OXP (Genencor International, Inc.). Suitable methods for making such protease mutants (oxidatively stable proteases), and particularly such mutants having a substitution for the methionine at a position equivalent to M222 in *B. amyloliquefaciens*, are described in US Re 34606. Suitable methods for determining

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"equivalent" positions in other subtilisins are provided in Re 34606, EP 257,446 and USSN 212,291, which are incorporated herein by reference.

Abbreviations used herein, particularly three letter or one letter notations for amino acids are described in Dale, J.W., Molecular Genetics of Bacteria, John Wiley & Sons, (1989) Appendix B.

Experimental

Example 1

Substitutions for the Methionine Residues in  
*B. licheniformis* Alpha-Amylase

The alpha-amylase gene (Fig. 1) was cloned from *B. licheniformis* NCIB8061 obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland (Gray, G. et al. (1986) J. Bacteriology 166:635-643). The 1.72kb PstI-SstI fragment, encoding the last three residues of the signal sequence; the entire mature protein and the terminator region was subcloned into M13MP18. A synthetic terminator was added between the BclI and SstI sites using a synthetic oligonucleotide cassette of the form:

**BclI**

**SstI**

5' GATCAAAACATAAAAACCGGCCCTGGCCCCGCCGGTTTTATTATTTGAGCT 3'  
3' TTTGTATTTTGGCCGGAACCGGGGGCGGCCAAAAATAATAAAAAC 5'

Seq ID No 1

designed to contain the *B. amyloliquefaciens* subtilisin transcriptional terminator (Wells et al. (1983) Nucleic Acid Research 11:7911-7925).

Site-directed mutagenesis by oligonucleotides used essentially the protocol of Zoller, M. et al. (1983) Meth. Enzymol. 100:468-500: briefly, 5'-phosphorylated oligonucleotide primers were used to introduce the desired mutations on the M13 single-stranded DNA template using the oligonucleotides listed in Table I to substitute

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for each of the seven methionines found in *B. licheniformis* alpha-amylase. Each mutagenic oligonucleotide also introduced a restriction endonuclease site to use as a screen for the linked mutation.

TABLE I

Mutagenic Oligonucleotides for the Substitution of the  
Methionine Residues in *B. licheniformis* Alpha-Amylase

<b>M8A</b>		
5'-T GGG ACG CTG <b>GCG</b> <u>CAG TAC TTT</u> GAA TGG T-3'	Seq ID No 2	
ScaI+		
<b>M15L</b>		
5'-TG ATG <b>CAG TAC TTT</b> GAA TGG <u>TAC CTG CCC AAT</u> GA-3'	Seq ID No 3	
ScaI+ KpnI+		
<b>M197L</b>		
5'-GAT TAT TTG TTG TAT GCC <u>GAT ATC GAC TAT GAC</u> CAT-3'	Seq ID No 4	
EcoRV+		
<b>M256A</b>		
5'-CG GGG AAG <b>GAG GCC</b> <u>TTT ACG GTA GCT</u> -3'	Seq ID No 5	
StuI+		
<b>M304L</b>		
5'-GC GGC TAT <b>GAC TTA AGG</b> AAA TTG C-3'	Seq ID No 6	
AfIII+		
<b>M366A</b>		
5'-C TAC GGG <b>GAT GCA</b> <u>TAC GGG ACG A</u> -3'	Seq ID No 7	
NsiI+		
<b>M366Y</b>		
5'-C TAC GGG GAT <b>TAC TAC GGG ACC AAG</b> GGA GAC TCC C-3'	Seq ID No 8	
StyI+		
<b>M438A</b>		
5'-CC GGT <b>GGG GCC AAG CGG GCC</b> TAT GTT GGC CGG CAA A-3'	Seq ID No 9	
SfiI+		

**Bold** letter indicate base changes introduced by oligonucleotide.

Codon changes indicated in the form M8A, where methionine (M) at position +8 has been changed to alanine (A).

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Underlining indicates restriction endonuclease site introduced by oligonucleotide.

The heteroduplex was used to transfect *E. coli* mutL cells (Kramer et al. (1984) Cell 38:879) and, after plaque-purification, clones were analyzed by restriction analysis of the RF1's. Positives were confirmed by dideoxy sequencing (Sanger et al. (1977) Proc. Natl. Acad. Sci. U.S.A. 74:5463-5467) and the PstI-SstI fragments for each subcloned into an *E. coli* vector, plasmid pA4BL.

Plasmid pA4BL

Following the methods described in US application 860,468 (Power et al.), which is incorporated herein by reference, a silent PstI site was introduced at codon +1 (the first amino-acid following the signal cleavage site) of the *aprE* gene from pS168-1 (Stahl, M.L. and Ferrari, E. (1984) J. Bacter. 158:411-418). The *aprE* promoter and signal peptide region was then cloned out of a pJH101 plasmid (Ferrari, F.A. et al. (1983) J. Bacter. 154:1513-1515) as a HindIII-PstI fragment and subcloned into the pUC18-derived plasmid JM102 (Ferrari, E. and Hoch, J.A. (1989) *Bacillus*, ed. C.R. Harwood, Plenum Pub., pp. 57-72). Addition of the PstI-SstI fragment from *B. licheniformis* alpha-amylase gave pA4BL (Fig. 5) having the resulting *aprE* signal peptide-amylase junction as shown in Fig. 6.

Transformation Into *B. subtilis*

pA4BL is a plasmid able to replicate in *E. coli* and integrate into the *B. subtilis* chromosome. Plasmids containing different variants were transformed into *B. subtilis* (Anagnostopoulos, C. and Spizizen, J. (1961) J. Bacter. 81:741-746) and integrated into the chromosome at the *aprE* locus by a Campbell-type mechanism (Young, M. (1984) J. Gen. Microbiol. 130:1613-1621). The *Bacillus subtilis* strain BG2473 was a derivative of I168 which had been deleted for amylase ( $\Delta$ amyE) and two proteases ( $\Delta$ apr,  $\Delta$ npr) (Stahl, M.L. and Ferrari, E., J. Bacter. 158:411-418 and US Patent 5,264,366, incorporated herein by reference). After transformation the *sacU32*(Hy) (Henner, D.J. et al. (1988) J. Bacter. 170:296-300)

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mutation was introduced by PBS-1 mediated transduction (Hoch, J.A. (1983) 154:1513-1515).

N-terminal analysis of the amylase expressed from pA4BL in *B. subtilis* showed it to be processed having four extra alanines at the N-terminus of the secreted amylase protein ("A4 form"). These extra residues had no significant, deleterious effect on the activity or thermal stability of the A4 form and in some applications may enhance performance. In subsequent experiments the correctly processed forms of the *licheniformis* amylase and the variant M197T were made from a very similar construction (see Fig. 6). Specifically, the 5' end of the A4 construction was subcloned on an EcoRI-SstII fragment, from pA4BL (Fig. 5) into M13BM20 (Boehringer Mannheim) in order to obtain a coding-strand template for the mutagenic oligonucleotide below:

5'-CAT CAG CGT CCC ATT AAG ATT TGC AGC CTG CGC AGA CAT GTT GCT-3'  
Seq ID No 10

This primer eliminated the codons for the extra four N-terminal alanines, correct forms being screened for by the absence of the PstI site. Subcloning the EcoRI-SstII fragment back into the pA4BL vector (Fig. 5) gave plasmid pBLapr. The M197T substitution could then be moved, on a SstII-SstI fragment, out of pA4BL (M197T) into the complementary pBLapr vector to give plasmid pBLapr (M197T). N-terminal analysis of the amylase expressed from pBLapr in *B. subtilis* showed it to be processed with the same N-terminus found in *B. licheniformis* alpha-amylase.

#### Example 2

##### Oxidative Sensitivity of Methionine Variants

*B. licheniformis* alpha-amylase, such as Spezyme® AA20 (commercially available from Genencor International, Inc.), is inactivated rapidly in the presence of hydrogen peroxide (Fig. 7). Various methionine variants were expressed in shake-flask cultures of *B. subtilis* and the crude supernatants purified by ammonium sulphate

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cuts. The amylase was precipitated from a 20% saturated ammonium sulphate supernatant by raising the ammonium sulphate to 70% saturated, and then resuspended. The variants were then exposed to 0.88M hydrogen peroxide at pH 5.0, at 25°C. Variants at six of the methionine positions in *B. licheniformis* alpha-amylase were still subject to oxidation by peroxide while the substitution at position +197 (M197L) showed resistance to peroxide oxidation. (See Fig. 7.) However, subsequent analysis described in further detail below showed that while a variant may be susceptible to oxidation at pH 5.0, 25°C, it may exhibit altered/enhanced properties under different conditions (i.e., liquefaction).

Example 3

Construction of All Possible Variants at Position 197

All of the M197 variants (M197X) were produced in the A4 form by cassette mutagenesis, as outlined in Fig. 8:

- 1) Site directed mutagenesis (via primer extension in M13) was used to make M197A using the mutagenic oligonucleotide below:

M197A  
5'-GAT TAT TTG GCG TAT GCC GAT ATC GAC TAT GAC CAT-3'  
EcoRV+  
ClaI- Seq ID No 11

which also inserted an EcoRV site (codons 200-201) to replace the ClaI site (codons 201-202).

- 2) Then primer LAAM12 (Table II) was used to introduce another silent restriction site (BstBI) over codons 186-188.
- 3) The resultant M197A (BstBI+, EcoRV+) variant was then subcloned (PstI-SstI fragment) into plasmid pA4BL and the resultant plasmid digested with BstBI and EcoRV and the large vector-containing fragment isolated by electroelution from agarose gel.

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4) Synthetic primers LAAM14-30 (Table II) were each annealed with the largely complementary common primer LAAM13 (Table II). The resulting cassettes encoded for all the remaining naturally occurring amino acids at position +197 and were ligated, individually, into the vector fragment prepared above.

**TABLE II**  
**Synthetic Oligonucleotides Used for Cassette Mutagenesis**  
**to Produce M197X Variants**

LAAM12	GG GAA GTT <u>TCG</u> <u>AAT</u> GAA AAC G	Seq ID No 12
LAAM13	X197bs (ECORV) <u>GTC</u> <u>GGC</u> <u>ATA</u> <u>TG</u> <u>CAT</u> ATA ATC ATA GTT GCC GTT TTC ATT (BstBI)	Seq ID No 13
LAAM14	I197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT TTG <u>ATC</u> TAT GCC GAC (ECORV-)	Seq ID No 14
LAAM15	F197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT TTG <u>TTC</u> TAT GCC GAC (ECORV-)	Seq ID No 15
LAAM16	V197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT TTG <u>GT</u> TAT GCC GAC (ECORV-)	Seq ID No 16
LAAM17	S197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT TTG <u>AGC</u> TAT GCC GAC (ECORV-)	Seq ID No 17
LAAM18	P197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT TTG <u>CCT</u> TAT GCC GAC (ECORV-)	Seq ID No 18
LAAM19	T197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT TTG <u>ACA</u> TAT GCC GAC (ECORV-)	Seq ID No 19
LAAM20	Y197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT TTG <u>TAC</u> TAT GCC GAC (ECORV-)	Seq ID No 20
LAAM21	H197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT TTG <u>CAC</u> TAT GCC GAC (ECORV-)	Seq ID No 21

LAAM22	G197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT <u>TTC</u> <u>GGC</u> TAT GCC GAC (ECoRV-)	Seq ID No 22
LAAM23	Q197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT <u>TTC</u> <u>CAA</u> TAT GCC GAC (ECoRV-)	Seq ID No 23
LAAM24	N197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT <u>TTC</u> <u>AAC</u> TAT GCC GAC (ECoRV-)	Seq ID No 24
LAAM25	K197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT <u>TTC</u> <u>AAA</u> TAT GCC GAC (ECoRV-)	Seq ID No 25
LAAM26	D197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT <u>TTC</u> <u>GAT</u> TAT GCC GAC (ECoRV-)	Seq ID No 26
LAAM27	E197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT <u>TTC</u> <u>GAA</u> TAT GCC GAC (ECoRV-)	Seq ID No 27
LAAM28	C197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT <u>TTC</u> <u>TGT</u> TAT GCC GAC (ECoRV-)	Seq ID No 28
LAAM29	W197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT <u>TTC</u> <u>TGG</u> TAT GCC GAC (ECoRV-)	Seq ID No 29
LAAM30	R197 (BstBI) CG AAT GAA AAC GGC AAC TAT GAT TAT <u>TTC</u> <u>AGA</u> TAT GCC GAC (ECoRV-)	Seq ID No 30

The cassettes were designed to destroy the EcoRV site upon ligation, thus plasmids from *E. coli* transformants were screened for loss of this unique site. In addition, the common bottom strand of the cassette contained a frame-shift and encoded a NsiI site, thus transformants derived from this strand could be eliminated by screening for the presence of the unique NsiI site and would not be expected, in any case, to lead to expression of active amylase.

Positives by restriction analysis were confirmed by sequencing and transformed in *B. subtilis* for expression in shake-flask cultures (Fig. 9). The specific activity of certain of the M197X mutants was then determined using a soluble substrate assay. The data generated using the following assay methods are presented below in Table III.

Soluble Substrate Assay: A rate assay was developed based on an end-point assay kit supplied by Megazyme (Aust.) Pty. Ltd.: Each vial of substrate (p-nitrophenyl maltoheptaoside, BPNPG7) was dissolved in 10ml of sterile water, followed by a 1 to 4 dilution in assay buffer (50mM maleate buffer, pH 6.7, 5mM calcium chloride, 0.002% Tween20). Assays were performed by adding 10 $\mu$ l of amylase to 790 $\mu$ l of the substrate in a cuvette at 25°C. Rates of hydrolysis were measured as the rate of change of absorbance at 410nm, after a delay of 75 seconds. The assay was linear up to rates of 0.4 absorption units/min.

The amylase protein concentration was measured using the standard Bio-Rad assay (Bio-Rad Laboratories) based on the method of Bradford, M. (1976) Anal. Biochem. 72:248) using bovine serum albumin standards.

Starch Hydrolysis Assay: The standard method for assaying the alpha-amylase activity of Spezyme® AA20 was used. This method is described in detail in Example 1 of USSN 07/785,624, incorporated herein by reference. Native starch forms a blue color with iodine but fails to do so when it is hydrolyzed into shorter dextrin molecules. The substrate is soluble Lintner starch 5gm/liter in phosphate buffer, pH 6.2 (42.5gm/liter potassium dihydrogen phosphate, 3.16gm/liter sodium hydroxide). The sample is added in 25mM calcium chloride and activity is measured as the time taken to give a negative iodine test upon incubation at 30°C. Activity is recorded in liquefons per gram or ml (LU) calculated according to the formula:

$$\text{LU/ml or LU/g} = \frac{570}{V \times t} \times D$$

Where LU=liquefon unit  
 V=volume of sample (5ml)  
 t=dextrinization time (minutes)  
 D=dilution factor=dilution volume/ml or g of added enzyme.

TABLE III

ALPHA-AMYLASE	SPECIFIC ACTIVITY (as % of AA20 value) on:	
	Soluble Substrate	Starch
Spezyme® AA20	100	100
A4 form	105	115
M15L (A4 form)	93	94
M15L	85	103
M197T (A4 form)	75	83
M197T	62	81
M197A (A4 form)	88	89
M197C	85	85
M197L (A4 form)	51	17

Example 4

Characterization of Variant M15L

Variant M15L made as per the prior examples did not show increased amylase activity (Table III) and was still inactivated by hydrogen peroxide (Fig. 7). It did, however, show significantly increased

performance in jet-liquefaction of starch, especially at low pH as shown in Table IV below.

Starch liquefaction was typically performed using a Hydroheater M 103-M steam jet equipped with a 2.5 liter delay coil behind the mixing chamber and a terminal back pressure valve. Starch was fed to the jet by a Moyno pump and steam was supplied by a 150 psi steam line, reduced to 90-100 psi. Temperature probes were installed just after the Hydroheater jet and just before the back pressure valve.

Starch slurry was obtained from a corn wet miller and used within two days. The starch was diluted to the desired solids level with deionized water and the pH of the starch was adjusted with 2% NaOH or saturated Na<sub>2</sub>CO<sub>3</sub>. Typical liquefaction conditions were:

Starch	32%-35% solids
Calcium	40-50 ppm (30 ppm added)
pH	5.0-6.0
Alpha-amylase	12-14 LU/g starch dry basis

Starch was introduced into the jet at about 350 ml/min. The jet temperature was held at 105°-107°C. Samples of starch were transferred from the jet cooker to a 95°C second stage liquefaction and held for 90 minutes.

The degree of starch liquefaction was measured immediately after the second stage liquefaction by determining the dextrose equivalence (DE) of the sample and by testing for the presence of raw starch, both according to the methods described in the Standard Analytical Methods of the Member Companies of the Corn Refiners Association, Inc., sixth edition. Starch, when treated generally under the conditions given above and at pH 6.0, will yield a liquefied starch with a DE of about 10 and with no raw starch. Results of starch liquefaction tests using mutants of the present invention are provided in Table IV.

TABLE IV

## Performance of Variants M15L (A4 form) and M15L in Starch Liquefaction

	<u>pH</u>	<u>DE after 90 Mins.</u>
Spezyme® AA20	5.9	9.9
M15L (A4 form)	5.9	10.4
Spezyme® AA20	5.2	1.2
M15L (A4 form)	5.2	2.2
Spezyme® AA20	5.9	9.3*
M15L	5.9	11.3*
Spezyme® AA20	5.5	3.25**
M15L	5.5	6.7**
Spezyme® AA20	5.2	0.7**
M15L	5.2	3.65**

\* average of three experiments

\*\* average of two experiments

## Example 5

## [ Construction of M15X Variants ]

Following generally the processes described in Example 3 above, all variants at M15 (M15X) were produced in native *B. licheniformis* by cassette mutagenesis, as outlined in Fig. 12:

1) Site directed mutagenesis (via primer extension in M13) was used to introduce unique restriction sites flanking the M15 codon to facilitate insertion of a mutagenesis cassette. Specifically, a BstB1 site at codons 11-13 and a Mscl site at codons 18-20 were introduced using the two oligonucleotides shown below:

M15XBstB1 5'-G ATG CAG TAT TTC GAA CTGG TAT A-3'  
BstB1 Seq ID No 48

M15XMscl 5'-TG CCC AAT GAT GGC CAA CAT TGG AAG-3'  
Mscl Seq ID No 49

2) The vector for M15X cassette mutagenesis was then constructed by subcloning the SfiI-SstII fragment from the mutagenized amylase (BstB1+, Mscl+) into plasmid pBLapr. The resulting plasmid was

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then digested with BstB1 and Msc1 and the large vector fragment isolated by electroelution from a polyacrylamide gel.

3) Mutagenesis cassettes were created as with the M197X variants. Synthetic oligomers, each encoding a substitution at codon 15, were annealed to a common bottom primer. Upon proper ligation of the cassette to the vector, the Msc1 is destroyed allowing for screening of positive transformants by loss of this site. The bottom primer contains an unique SnaB1 site allowing for the transformants derived from the bottom strand to be eliminated by screening for the SnaB1 site. This primer also contains a frameshift which would also eliminate amylase expression for the mutants derived from the common bottom strand.

The synthetic cassettes are listed in Table V and the general cassette mutagenesis strategy is illustrated in Figure 12.

TABLE V  
Synthetic Oligonucleotides Used for Cassette Mutagenesis  
to Produce M15X Variants

M15A (BstB1) C GAA TGG TAT <u>GCT</u> CCC AAT GAC GG (Mscl)	Seq ID No 50
M15R (BstB1) C GAA TGG TAT <u>CGC</u> CCC AAT GAC GG (Mscl)	Seq ID No 51
M15N (BstB1) C GAA TGG TAT <u>AAT</u> CCC AAT GAC GG (Mscl)	Seq ID No 52
M15D (BstB1) C GAA TGG TAT <u>GAT</u> CCC AAT GAC GG (Mscl)	Seq ID No 53
M15H (BstB1) C GAA TGG TAT <u>CAC</u> CCC AAT GAC GG (Mscl)	Seq ID No 54
M15K (BstB1) C GAA TGG TAT <u>AAA</u> CCC AAT GAC GG (Mscl)	Seq ID No 55
M15P (BstB1) C GAA TGG TAT <u>CCG</u> CCC AAT GAC GG (Mscl)	Seq ID No 56
M15S (BstB1) C GAA TGG TAT <u>TCT</u> CCC AAT GAC GG (Mscl)	Seq ID No 57
M15T (BstB1) C GAA TGG TAC <u>ACT</u> CCC AAT GAC GG (Mscl)	Seq ID No 58
M15V (BstB1) C GAA TGG TAT <u>GTT</u> CCC AAT GAC GG (Mscl)	Seq ID No 59
M15C (BstB1) C GAA TGG TAT <u>TGT</u> CCC AAT GAC GG (Mscl)	Seq ID No 60
M15Q (BstB1) C GAA TGG TAT <u>CAA</u> CCC AAT GAC GG (Mscl)	Seq ID No 61
M15E (BstB1) C GAA TGG TAT <u>GAA</u> CCC AAT GAC GG (Mscl)	Seq ID No 62
M15G (BstB1) C GAA TGG TAT <u>GGT</u> CCC AAT GAC GG (Mscl)	Seq ID No 63
M15I (BstB1) C GAA TGG TAT <u>ATT</u> CCC AAT GAC GG (Mscl)	Seq ID No 64
M15F (BstB1) C GAA TGG TAT <u>TTT</u> CCC AAT GAC GG (Mscl)	Seq ID No 65
M15W (BstB1) C GAA TGG TAC <u>TGG</u> CCC AAT GAC GG (Mscl)	Seq ID No 66
M15Y (BstB1) C GAA TGG TAT <u>TAT</u> CCC AAT GAC GG (Mscl)	Seq ID No 67
M15X (Mscl) CC GTC ATT GGG ACT ACG TAC CAT T (BstB1) (bottom strand)	Seq ID No 68

Underline indicates codon changes at amino acid position 15.

Conservative substitutions were made in some cases to prevent introduction of new restriction sites.

Example 6Bench Liquefaction with M15X Variants

Eleven alpha-amylase variants with substitutions for M15 made as per Example 5 were assayed for activity, as compared to Spezyme® AA20 (commercially available from Genencor International, Inc.) in liquefaction at pH 5.5 using a bench liquefaction system. The bench scale liquefaction system consisted of a stainless steel coil (0.25 inch diameter, approximately 350 ml volume) equipped with a 7 inch long static mixing element approximately 12 inches from the anterior end and a 30 psi back pressure valve at the posterior end. The coil, except for each end, was immersed in a glycerol-water bath equipped with thermostatically controlled heating elements that maintained the bath at 105-106°C.

Starch slurry containing enzyme, maintained in suspension by stirring, was introduced into the reaction coil by a piston driven metering pump at about 70 ml/min. The starch was recovered from the end of the coil and was transferred to the secondary hold (95°C for 90 minutes). Immediately after the secondary hold, the DE of the liquefied starch was determined, as described in Example 4. The results are shown in Fig. 16.

Example 7Characterization of M197X Variants

As can be seen in Fig. 9, there was a wide range of amylase activity (measured in the soluble substrate assay) expressed by the M197X (A4 form) variants. The amylases were partially purified from the supernatants by precipitation with two volumes of ethanol and resuspension. They were then screened for thermal stability (Fig. 10) by heating at 95°C for 5 minutes in 10mM acetate buffer pH 5.0, in the presence of 5mM calcium chloride; the A4 wild-type retained 28% of its activity after incubation. For M197W and M197P we were unable to recover active protein from the supernatants. Upon sequencing, the M197H variant was found to contain a second mutation, N190K. M197L was examined in a separate experiment and was one of the lowest thermally stable variants. There appears to

be a broad correlation between expression of amylase activity and thermal stability. The *licheniformis* amylase is restricted in what residues it can accommodate at position 197 in terms of retaining or enhancing thermal stability: cysteine and threonine are preferred for maximal thermal stability under these conditions whereas alanine and isoleucine are of intermediate stability. However, other substitutions at position +197 result in lowered thermal stability which may be useful for other applications. Additionally, different substitutions at +197 may have other beneficial properties, such as altered pH performance profile or altered oxidative stability. For example, the M197C variant was found to inactivate readily by air oxidation but had enhanced thermal stability. Conversely, compared to the M197L variant, both M197T and M197A retained not only high thermal stability (Fig. 10), but also high activity (Table III), while maintaining resistance to inactivation by peroxide at pH 5 to pH 10 (Fig. 7).

#### Example 8

##### Stability and Performance in Detergent Formulation

The stability of the M197T (A4 form), M197T and M197A (A4 form) was measured in automatic dish care detergent (ADD) matrices. 2ppm Savinase™ (a protease, commercially available from Novo Industries, of the type commonly used in ADD) were added to two commercially available bleach-containing ADD's: Cascade™ (Procter and Gamble, Ltd.) and Sunlight™ (Unilever) and the time course of inactivation of the amylase variants and Termamyl™ (a thermally stable alpha-amylase available from Novo Nordisk, A/S) followed at 65°C. The concentration of ADD product used in both cases was equivalent to 'pre-soak' conditions: 14gm product per liter of water (7 grams per gallon hardness). As can be seen (Figs. 11a and 11b), both forms of the M197T variant were much more stable than Termamyl™ and M197A (A4 form), which were inactivated before the first assay could be performed. This stability benefit was seen in the presence or absence of starch as determined by the following protocol. Amylases were added to 5ml of ADD and Savinase™,

prewarmed in a test tube and, after vortexing, activities were assayed as a function of time, using the soluble substrate assay. The "+ starch" tube had spaghetti starch baked onto the sides (140°C, 60 mins.). The results are shown in Figs. 11a and 11b.

Example 9

Characterization of M15X Variants

All M15X variants were propagated in *Bacillus subtilis* and the expression level monitored as shown in Fig. 13. The amylase was isolated and partially purified by a 20-70% ammonium sulfate cut. The specific activity of these variants on the soluble substrate was determined as per Example 3 (Fig. 14). Many of the M15X amylases have specific activities greater than that of Spezyme® AA20. A benchtop heat stability assay was performed on the variants by heating the amylase at 90°C for 5 min. in 50 mM acetate buffer pH 5 in the presence of 5 mM CaCl<sub>2</sub> (Fig. 15). Most of the variants performed as well as Spezyme® AA20 in this assay. Those variants that exhibited reasonable stability in this assay (reasonable stability defined as those that retained at least about 60% of Spezyme® AA20's heat stability) were tested for specific activity on starch and for liquefaction performance at pH 5.5. The most interesting of those mutants are shown in Fig. 16. M15D, N and T, along with L, outperformed Spezyme® AA20 in liquefaction at pH 5.5 and have increased specific activities in both the soluble substrate and starch hydrolysis assays.

Generally, we have found that by substituting for the methionine at position 15, we can provide variants with increased low pH-liquefaction performance and/or increased specific activity.

Example 10

Tryptophan Sensitivity to Oxidation

Chloramine-T (sodium N-chloro-p-toluenesulfonimide) is a selective oxidant, which oxidizes methionine to methionine sulfoxide at neutral or alkaline pH. At acidic pH, chloramine-T will modify both methionine and tryptophan (Schechter, Y., Burstein, Y. and

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Patchornik, A. (1975) Biochemistry 14 (20) 4497-4503). Fig. 17 shows the inactivation of *B. licheniformis* alpha-amylase with chloramine-T at pH 8.0 (AA20 = 0.65 mg/ml, M197A = 1.7 mg/ml, M197L = 1.7 mg/ml). The data shows that by changing the methionine at position 197 to leucine or alanine, the inactivation of alpha-amylase can be prevented. Conversely, as shown in Fig. 18, at pH 4.0 inactivation of the M197A and M197L proceeds, but require more equivalents of chloramine-T (Fig. 18; AA20 = 0.22 mg/ml, M197A = 4.3 mg/ml, M197L = 0.53 mg/ml; 200 mM NaAcetate at pH 4.0). This suggests that a tryptophan residue is also implicated in the chloramine-T mediated inactivation event. Furthermore, tryptic mapping and subsequent amino acid sequencing indicated that the tryptophan at position 138 was oxidized by chloramine-T (data not shown). To prove this, site-directed mutants were made at tryptophan 138 as provided below:

Preparation of Alpha-Amylase Double Mutants W138 and M197\*

Certain variants of W138 (F, Y and A) were made as double mutants, with M197T (made as per the disclosure of Example 3). The double mutants were made following the methods described in Examples 1 and 3. Generally, single negative strands of DNA were prepared from an M13MP18 clone of the 1.72kb coding sequence (Pst I-Sst I) of the *B. licheniformis* alpha-amylase M197T mutant. Site-directed mutagenesis was done using the primers listed below, essentially by the method of Zoller, M. et al. (1983) except T4 gene 32 protein and T4 polymerase were substituted for klenow. The primers all contained unique sites, as well as the desired mutation, in order to identify those clones with the appropriate mutation.

Tryptophan 138 to Phenylalanine

133 134 135 136 137 138 139 140 141 142 143  
CAC CTA ATT AAA GCT TTC ACA CAT TTT CAT TTT  
Hind III

Seq ID No 42

Tryptophan 138 to Tyrosine

133 134 135 136 137 138 139 140 141 142 143  
CAC CTA ATT AAA GCT TAC ACA CAT TTT CAT TTT  
Hind III

Seq ID No 43

Tryptophan 138 to Alanine - This primer also engineers unique sites upstream and downstream of the 138 position.

127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142  
C CGC GTA ATT TCC GGA GAA CAC CTA ATT AAA GCC GCA ACA CAT TTT CAT  
BspE I

143 144 145 146 147  
TTT CCC GGG CGC GGC AG  
Xma I

Seq ID No 44

Mutants were identified by restriction analysis and W138F and W138Y confirmed by DNA sequencing. The W138A sequence revealed a nucleotide deletion between the unique BspE I and Xma I sites, however, the rest of the gene sequenced correctly. The 1.37kb SstII/SstI fragment containing both W138X and M197T mutations was moved from M13MP18 into the expression vector pBLapr resulting in pBLapr (W138F, M197T) and pBLapr (W138Y, M197T). The fragment containing unique BspE I and Xma I sites was cloned into pBLapr (BspE I, Xma I, M197T) since it is useful for cloning cassettes containing other amino acid substitutions at position 138.

Single Mutations at Amino Acid Position 138

Following the general methods described in the prior examples, certain single variants of W138 (F, Y, L, H and C) were made.

The 1.24kb Asp718-SstI fragment containing the M197T mutation in plasmid pBLapr (W138X, M197T) of Example 7 was replaced by the wild-type fragment with methionine at 197, resulting in pBLapr (W138F), pBLapr (W138Y) and pBLapr (BspE I, Xma I).

The mutants W138L, W138H and W138C were made by ligating synthetic cassettes into the pBLapr (BspE I, Xma I) vector using the following primers:

Tryptophan 138 to Leucine

CC GGA GAA CAC CTA ATT AAA GCC CTA ACA CAT TTT CAT TTT C

Seq ID No 45

Tryptophan 138 to HistidineCC GGA GAA CAC CTA ATT AAA GCC **CAC** ACA CAT TTT CAT TTT C

Seq ID No 46

Tryptophan 138 to CysteineCC GGA GAA CAC CTA ATT AAA GCC **TGC** ACA CAT TTT CAT TTT C

Seq ID No 47

Reaction of the double mutants M197T/W138F and M197T/W138Y with chloramine-T was compared with wild-type (AA20 = 0.75 mg/ml, M197T/W138F = 0.64 mg/ml, M197T/W138Y = 0.60 mg/ml; 50 mM NaAcetate at pH 5.0). The results shown in Fig. 19 show that mutagenesis of tryptophan 138 has caused the variant to be more resistant to chloramine-T.

Example 11Preparation of Multiple Mutants\*

Following the methods of Examples 1, 3, 5 and 10, the following multiple mutants were made: M15T/M197T; M15S/M197T; W138Y/M197T; M15S/W138Y/M197T and M15T/W138Y/M197T. Certain of these multiple mutants were previously exemplified, for example, W138Y/M197T was made and tested in Example 10. The multiple mutants were identified by restriction analysis.

Various multiple mutants within the scope of the present invention were further tested for performance as cleaning products (automatic dish care detergent) additives. These tests are detailed below.

Stability Testing

A 4000 ppm solution of automatic dishwashing detergent (ADD) containing perborate and TAED was prepared in water with a hardness of 7 gpg. Certain amylase mutants described above were added to this ADD solution to yield a rate of 0.4 when assayed by the Ceralpha method (Megazyme (Austr.) Pty. Ltd., Parramatta, NSW, Australia). One set of samples was held at room temperature (21-

23°C) for about 30 min. (non-heated). A second set of samples was warmed from room temperature to about 65°C after addition of the enzyme (heated). 30 min. after addition of the enzyme, the activity of the amylase mutants was measured and the activity relative to the activity at the time of addition of the enzyme was calculated (relative activity %).

The results shown in Fig. 20 indicate that the methionine at position +197 of *B. licheniformis* alpha-amylase should be modified for stability in a formulation comprising ADD + perborate + TAED.

#### Starch Hydrolysis Assay

A 4000 ppm solution of automatic dishwashing detergent (ADD) containing perborate and TAED was prepared in water with a hardness of 7 gpg and three cooked pieces of elbow macaroni were added. The amylase mutants described above were added to this ADD solution to yield a final concentration of 5 ppm active enzyme. The tubes were incubated at 50°C for about 30 min. and the concentration of reducing sugars released was measured against a glucose standard curve using the dinitrosalicylic acid method. Results are shown in Table VI.

Table VI

Enzyme	Reducing Sugar Concentration (g/l)	Standard Deviation
No Enzyme	1.64	0.12
Wild-Type	4.97	0.30
M15S/M197T	5.40	0.36
M15T/M197T	5.85	0.38
W138Y/M197T	6.48	0.36
M15S/W138Y/M197T	6.04	0.74
M15T/W138Y/M197T	6.27	0.49

The results shown in Table VI show that M15T/M197T; M15S/M197T; W138Y/M197T; M15S/W138Y/M197T and M15T/W138Y/M197T performed well compared to no enzyme and wild-type alpha-amylase controls.

#### Oatmeal Stains

Dishes were evenly soiled with a cooked, blended oatmeal paste and dried overnight at 37°C. Dishes were loaded in an ASKO Model 770 dishwasher and washed at 45°C on the Quick Wash cycle using 10 g of automatic dishwashing detergent containing 5% perborate, 3% TAED and 11 mg of certain amylase enzyme(s). The plates were weighed before soiling, after soiling and after washing, and the average % soil removed from all plates was calculated. The data are shown below in Table VII.

Table VII

Enzyme	% Soil Removed (Average of All Dishes)
Wild-Type	61
M15S/M197T	66
M15T/M197T	71
W138Y/M197T	68
M15S/W138Y/M197T	62
M15T/W138Y/M197T	72

The data show that the mutant enzymes provided a benefit greater than that provided by the wild-type. Wild-type amylase provided a 20% greater cleaning benefit in removing oatmeal than did ADD without amylase.

Example 12  
Dish Care Cleaning Composition

1% (w/w) granules of wild-type and mutant amylases were formulated with a Korex Automatic Dishwasher Detergent to which 5% (w/w) sodium perborate monohydrate and 3% (w/w) TAED were added. Samples of these formulations were placed at room temperature (21-23°C) or at 38°C and 80% relative humidity for four weeks. Results are shown in Figs. 21 and 22.

The data show that the wild-type amylase activity, as measured by the Ceralpha method, decreased with increasing storage time in detergent. At room temperature, the mutant enzymes were completely stable. At 38°C and 80% relative humidity, all mutants were more stable than the wild-type.

The advantage of formulating an automatic dishwashing detergent with these mutant amylases is that these mutants are significantly more stable than the wild-type in the presence of perborate and

TAED and they provide a significant performance benefit in removing starchy food stains in the wash.

Example 13

Oxidatively Stable Protease/  
Oxidatively Stable Amylase Stability Studies

Enzyme granules containing either: 1) wild-type protease and wild-type amylase; or 2) bleach stable protease (GG36-M222S) made by the methods described in US Re 34606 and bleach stable amylase (AA20-M15T/W138Y/M197T) were dissolved in buffer containing 0.1 M sodium borate pH 10.2 and 0.005% Tween 80 at a concentration of 12.5 mg of each enzyme. To 9 ml of these solutions was added either 1 ml distilled water or 1 ml 30% hydrogen peroxide. After incubation of the solutions at 25°C for 30 minutes, the protease and amylase activity in each solution was measured and is reported as % of the original activity. The data are shown below in Table VIII.

Table VIII

Treatment	Enzyme	% Activity After 30 Min
Water	WT Amylase	104
Water	WT Protease	94
Water	M222S Protease	119
Water	TYT Amylase	88
3% Peroxide	WT Amylase	14
3% Peroxide	WT Protease	7
3% Peroxide	M222S Protease	116
3% Peroxide	TYT Amylase	75

The data show that the combination of a bleach-stable amylase mutant and a bleach-stable protease mutant, both with mutations at amino acid residues sensitive to oxidation, provides the combined benefits of protease and amylase in a formulation resistant to inactivation by bleach. The combination of a bleach-stable amylase and a bleach-stable protease retains most of its initial activity after 30 minutes in bleach, while the combination of wild-type

enzymes loses over 80% of its initial activity in the same period of time.

Example 14

Comparative Activity Profile of Amylase Variants

The activity profiles of amylase variants were obtained. Two Phadebas tablets (Phadebas Amylase Test Kit, Pharmacia Diagnostics) were dissolved in separate vials of 12 milliliters of buffer (0.05M borate/0.05M potassium phosphate/0.005M calcium chloride) at pH of about 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 to determine relative starch hydrolysis activity of each tested enzyme. After addition of the two tablets, the resultant pH values were 6.21, 7.0, 7.68, 8.75, 9.72 and 10.63. Each vial was mixed by magnetic stirring and, while mixing, 200 microliter aliquots were pipetted into a 96 well Costar polystyrene plate. Enzyme samples were obtained from solutions containing 9.9 mg/ml of Termamyl® (wild type *Bacillus licheniformis* alpha amylase available from Novo Nordisk, Denmark), 5.2 mg/ml Spezyme® AA20 (*Bacillus licheniformis* alpha amylase available from Genencor International, Inc., South San Francisco Ca.) or 4.1 mg/ml mutant amylase according to the invention (M15T/W138Y/M197T). Each sample was diluted to 1/5000 into an amylase assay buffer (50 mM acetate buffer, pH 6.7, 5mM CaCl<sub>2</sub>, .002% Tween 20. Ten microliters of diluted enzyme were added to the substrate solution by multichannel pipette. The reaction mixture was mixed at 1100 rpm using an Ika-Schuttler MTS 2 plate shaker for 30 minutes. The reaction was terminated by filtering insoluble substrate particles from supernatant containing enzyme and solubilized blue dye using 0.45 micron hydrophilic 96 well filtration plates (available in the Multiscreen Filtration System from Millipore, Bedford, Mass.). The samples were removed with a multichannel pipette. A 200 microliter control was used for each pH containing substrate with no enzyme.

From the filtered supernatant, 100 microliter aliquots of each sample were drawn into another 96 well Costar polystyrene plate, again by multichannel pipette, and the absorbance was read at 620nm. The results are shown in Figure 23 by plotting percent

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activity vs. incubation time. As shown in Figure 23, the mutant enzyme according to the present invention possesses superior activity at a pH range of from at least 6 to about 8.5 when compared to Termamyl® or Spezyme®AA20.

Example 15

Oxidative Stability of Amylase to Peracetic Acid

12.3 microliters of amylase was diluted into an Eppendorf tube containing 977.7 microliters of a solution of 0.1M CHES (2-n-cyclohexylaminoethane-sulfonic acid) and 0.005M calcium chloride at a pH of 10.0 at 25°C. The sample was mixed by vortexing and a 10 microliter aliquot was removed to determine initial activity determination on maltoheptaoside. The remaining 980 microliters were combined with 10 microliters of 32% peracetic acid. During the test, the samples were incubated in a heating block with glycerol as the conducting fluid. The temperature was 52°C as measured inside of the reaction tube. The caps of the tubes were sealed during the reaction. The pH of the buffer at 52°C was approximately 9.3, a value consistent with the expected pH shift for CHES buffer at higher temperature as reported in Methods of Enzymology, volume 87 (1982).

The sample was vortexed and 10 milliliter aliquots removed for activity determination at 0, 15, 30, 45 and 60 minutes. The results are shown in Figure 24 by plotting the relative activity (based on the release of hydrolyzed starch into solution) vs. pH. As shown in Figure 24, the mutant enzyme according to the present invention retains an exceptionally high percentage of its initial activity when compared to Termamyl® when incubated with an oxidant at a pH of 9.3.

Example 16

Comparative Wash Performance of Amylase in Liquid Laundry Detergent

The wash performance ability of mutant amylase according to the invention in typical liquid laundry detergent conditions in comparison with commercially available wild type amylase was tested. Corn starch/india ink soiled cotton swatches PEPD 7435WRL

(obtained from Scientific Services S/D, Inc., Sparrow Bush, N.Y.) were used for testing wash performance of amylase additives. Grease releasing TIDE® concentrated detergent (available commercially and from Proctor & Gamble, Cincinnati Ohio) was heated at 90°C for one hour to inactivate any enzymes present (e.g., protease or lipase). Amylases used were the Termamyl® 60T amylase (wild type *Bacillus licheniformis* amylase available from Novo Nordisk, Denmark) and the inventive amylase, M15T/W138Y/M197T.

1.0 gram aliquots of liquid detergent and amylase sufficient to result in 0.05, 0.1, 0.5, or 1.0 ppm of amylase in the final wash solution (either Termamyl® or M15T/W138Y/M197T) were measured and set aside. A control was performed with no amylase added. Wash tests were performed in a Model 7243S Terg-o-tometer (United States Testing Co., Hoboken, N.J.) The wash liquor comprised distilled water with 10 ml of hard water concentrate added to result in a final concentration of 100 ppm  $\text{Ca}^{2+}$  ion and 50 ppm  $\text{Mg}^{2+}$ . One liter of wash liquor was added to each Terg-o-tometer pot. When the wash liquor reached the appropriate temperature, the agitators were turned on and the detergent and amylase were simultaneously added to the wash liquor. After allowing 3 minutes for dissolution, the swatches were added to the wash liquor. After 15 minutes of agitation at 100rpm, the agitators were turned off and the swatches removed from the Terg-o-tometer pots and rinsed in a washing machine with cold water for 10 minutes. After rinsing, the starch swatches were removed and pressed dry. The swatches were then read with a reflectometer to determine the amount of cleaning. Trials were performed at both 40°C and 55°C. The results are shown in Figures 25 and 26, respectively, plotting the change in reflectance (normalized for detergent alone) vs. ppm amylase added.

As shown in Figures 25 and 26, the mutant amylase of the present invention significantly outperforms commercially available amylase in cleaning ability.

Example 17

Wash Performance of Inventive Amylase in Liquid Detergents

The wash performance of mutant amylase according to the present invention was tested in combination with two commercially available liquid detergent compositions, SA8® (commercially available from Amway Corp., Ada, Michigan) and Purex® (commercially available from the Dial Corp., Phoenix, Arizona). Mutant amylase (M15T/W138Y/M197T) was added to final concentrations in the wash liquor of 0.0, 0.05, 0.1, 0.5, 1.0 and 5.0 ppm. SA8® was added to the wash liquor in a quantity of 0.75gram/liter to bring the wash solution to a pH of 6.5 and a temperature of 55°C. Purex® was added to the wash liquor in a quantity of 1.0 gram/liter to bring the wash solution to a pH of 9.0 and a temperature of 40°C. Wash procedure was otherwise as in Example 16. The swatches were measured to determine the change in reflectance (normalized for detergent alone). The results are given in Figure 27.

As can be seen in Figure 27, the addition of amylase significantly improved the cleaning ability of the detergents.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: GENENCOR INTERNATIONAL, INC.

(ii) TITLE OF INVENTION: An Improved Laundry Detergent Composition Comprising Amylase

(iii) NUMBER OF SEQUENCES: 68

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- (F) ZIP: 94080

## (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Stone, Christopher L.
- (B) REGISTRATION NUMBER: 35,696
- (C) REFERENCE/DOCKET NUMBER: GC220-4

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- (B) TELEFAX: (415) 742-7217

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 56 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GATCAAAACA TAAAAAAACCG GCCTTGGCCC CGCCGGTTTT TTATTATTTT TGAGCT

56

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGGGACGCTG CGCGAGTACT TTGAATGGT

29

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGATGCAGTA CTTTGAATGG TACCTGCCCA ATGA

34

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 36 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATTATTTGT TGTATGCCGA TATCGACTAT GACCAT

36

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGGGGAACGA GGCCTTACG GTAGCT

26

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGGCTATGA CTTAAGGAAA TTGC

24

## (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTACGGGGAT GCATAACGGGA CGA

23

## (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 35 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTACGGGGAT TACTACGGGA CCAAGGGAGA CTCCC

35

## (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCGGTGGGGC CAAGCGGGCC TATGTTGGCC GGCAAA

36

## (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 45 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CATCAGCGTC CCATTAAGAT TTGCAGCCTG CGCAGACATG TTGCT

45

## (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

-- 47 --

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GATTATTTGG CGTATGCCGA TATCGACTAT GACCAT

36

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGGAAGTTTC GAATGAAAAC G

21

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCGGCATAT GCATATAATC ATAGTTGCCG TTTTCATT

38

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGAATGAAAA CGGCAACTAT GATTATTTGA TCTATGCCGA C

41

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGAATGAAAA CGGCAACTAT GATTATTTGT TCTATGCCGA C

41

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGAATGAAAA CGGCAACTAT GATTATTTGG TTTATGCCGA C

41

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGAATGAAAA CGGCAACTAT GATTATTTGA GCTATGCCGA C

41

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGAATGAAAA CGGCAACTAT GATTATTTGC CTTATGCCGA C

41

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGAATGAAAA CGGCAACTAT GATTATTTGA CATATGCCGA C

41

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CGAATGAAAA CGGCAACTAT GATTATTTGT ACTATGCCGA C

41

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGAATGAAAA CGGCAACTAT GATTATTTGC ACTATGCCGA C

41

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGAATGAAAA CGGCAACTAT GATTATTTGG GCTATGCCGA C

41

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CGAATGAAAA CGGCAACTAT GATTATTTGC AATATGCCGA C

41

## (2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 41 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CGAATGAAAA CGGCAACTAT GATTATTTGA ACTATGCCGA C

41

## (2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 41 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GCAATGAAAA CGGCAACTAT GATTATTTGA AATATGCCGA C

41

## (2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 41 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CGAATGAAAA CGGCAACTAT GATTATTTGG ATTATGCCGA C

41

## (2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 41 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CGAATGAAAA CGGCAACTAT GATTATTTGG AATATGCCGA C

41

## (2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

-- 51 --

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGAATGAAAA CGGCAACTAT GATTATTTGT GTATTGCCGA C

41

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CGAATGAAAA CGGCAACTAT GATTATTTGT GGTATGCCGA C

41

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CGAATGAAAA CGGCAACTAT GATTATTTGA GATATGCCGA C

41

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1968 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AGCTTGAAAGA AGTGAAGAAG CAGAGAGGCT ATTGAATAAA TGAGTAGAAA GCGCCATATC	60
GGCGCTTTTC TTTTGAAGA AAATATAGGG AAAATGGTAC TTGTTAAAAA TTCGGAATAT	120
TTATACAACA TCATATGTTT CACATTGAAA GGGGAGGAGA ATCATGAAAC AACAAAAACG	180
GCTTTACGCC CGATTGCTGA CGCTGTTATT TGCGCTCATC TTCTTGCTGC CTCATTCTGC	240
AGCAGCGGCG GCAAATCTTA ATGGGACGCT GATGCAGTAT TTTGAATGGT ACATGCCAA	300

TGACGGCAA CATTGGAAGC	360
TTTGCAAAA CGACTCGCA	
TATTTGGCTG AACACGGTAT	
TA	
CTCGCTC TGGATTCCCC CGGCATATAA	420
GGGAACGAGC CAAGCGGATG	
TGGGCTACGG	
TGCTTACGAC CTTTATGATT TAGGGGAGTT	480
TCATCAAAA GGGACGGTTC	
GGACAAAGTA	
CGGCACAAA GGAGAGCTGC	540
AATCTGCGAT CAAAAGTCTT	
CATTCCCGCG ACATTAACGT	
TTACGGGAT GTGGTCATCA	600
ACCACAAAGG CGGCCTGAT	
GCGACCGAAG ATGTAACCGC	
GGTGAAAGTC GATCCCGCTG	660
ACCGCAACCG CGTAATTCA	
GGAGAACACC TAATTAAAGC	
CTGGACACAT TTTCATTTTC	720
CGGGCGCGG CAGCACATAC	
AGCGATTITA AATGGCATTG	
GTACCATTTT GACGGAACCG	780
ATTGGGACGA GTCCCGAAAG	
CTGAACCGCA TCTATAAGTT	
TCAAGGAAG GCTTGGGATT	840
GGGAAGTTTC CAATGAAAC	
GGCAACTATG ATTATTTGAT	
GTATGCCAC ATCGATTATG	900
ACCATCCTGA TGCGCAGCA	
GAAATTAAGA GATGGGGCAC	
TTGGTATGCC AATGAACCTGC	960
AATTGGACGG TTTCCGCTT	
GATGCTGTCA AACACATTAA	
ATTTTCTTT TTGCGGGATT	1020
GGGTTAATCA TGTCAGGGAA	
AAAACGGGAA AGGAAATGTT	
TACGGTAGCT GAATATTGGC	1080
AGAATGACTT GGGCGCGCTG	
GAAAACATT TGAACAAAAC	
AAATTTAAAT CATTCACTGT	1140
TTGACGTGCC GCTTCATTAT	
CAGTCCATG CTGCATCGAC	
ACAGGGAGGC GGCTATGATA	1200
TGAGGAATT GCTGAACGGT	
ACGGTCGTTT CCAAGCATCC	
GTTGAATCG TTACATTTG	1260
TCGATAACCA TGATACACAG	
CCGGGGCAAT CGCTTGAGTC	
GACTGTCAA ACATGGTTA	1320
AGCCGTTGC TTACGTTTT	
ATTCTCACAA GGGATCTGG	
ATACCCCTCAG GTTTCTACG	1380
GGGATATGTA CGGGACGAAA	
GGAGACTCCC AGCGCGAAAT	
TCCTGCCCTG AAACACAAA	1440
TTGAACCGAT CTTAAAGCG	
AGAAAACAGT ATGCGTACGG	
AGCACAGCAT GATTATTCG	1500
ACCACCATGA CATTGTCGGC	
TGGACAAGGG AAGGCGACAG	
CTCGGTTGCA AATTCAAGTT	1560
TGGCGGCATT AATAACAGAC	
GGACCCGGTG GGGCAAGCG	
AATGTATGTC GGCCGGAAA	1620
ACGCCGGTGA GACATGGCAT	
GACATTACCG GAAACCGTTC	
GGAGCCGGTT GTCATCAATT	1680
CGGAAGGCTG GGGAGAGTTT	
CACGTAAACG GCGGGTCGGT	
TTCAATTAT GTTCAAAGAT	1740
AGAAGAGCAG AGAGGACGGA	
TTTCTGAAG GAAATCCGTT	
TTTTTATTTT GCCCGTCTTA	1800
TAAATTTCTT TGATTACATT	
TTATAATTAA TTAAACAAA	
GTGTCACTAG CCCTCAGGAA	1860
GGACTTGCTG ACAGTTGAA	
TCGCATAGGT AAGGGGGGA	
TGAAATGGCA ACGTTATCTG	1920
ATGTAGCAA GAAAGCAAAT	
GTGTGAAA TGACGGTATC	
GCAGGTGATC AATCATCCTG	1968
AGACTGTGAC GGATGAATTG	
AAAAAGCT	

## (2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 483 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro  
 1 5 10 15

Asn Asp Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu  
 20 25 30

Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly  
 35 40 45

Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu  
 50 55 60

Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys  
 65 70 75 80

Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn  
 85 90 95

Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr  
 100 105 110

Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val  
 115 120 125

Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro  
 130 135 140

Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe  
 145 150 155 160

Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys  
 165 170 175

Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn  
 180 185 190

Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val  
 195 200 205

Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln  
 210 215 220

Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe  
 225 230 235 240

Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met  
 245 250 255

Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn  
 260 265 270

Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu  
 275 280 285

His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Tyr Asp Met  
 290 295 300

Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser  
 305 310 315 320

Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu  
 325 330 335

Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu  
 340 345 350

Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly  
 355 360 365  
 Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile  
 370 375 380  
 Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His  
 385 390 395 400  
 Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp  
 405 410 415  
 Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro  
 420 425 430  
 Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr  
 435 440 445  
 Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser  
 450 455 460  
 Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr  
 465 470 475 480  
 Val Gln Arg

## (2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 511 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe  
 1 5 10 15  
 Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Asn Leu  
 20 25 30  
 Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly  
 35 40 45  
 His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His Gly  
 50 55 60  
 Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln Ala  
 65 70 75 80  
 Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe His  
 85 90 95  
 Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu Gln  
 100 105 110  
 Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly Asp  
 115 120 125  
 Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val Thr  
 130 135 140

Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly Glu  
 145 150 155 160  
 His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Arg Gly Ser  
 165 170 175  
 Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr Asp  
 180 185 190  
 Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly Lys  
 195 200 205  
 Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr Leu  
 210 215 220  
 Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu Ile  
 225 230 235 240  
 Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly Phe  
 245 250 255  
 Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp Trp  
 260 265 270  
 Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val Ala  
 275 280 285  
 Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn Lys  
 290 295 300  
 Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln Phe  
 305 310 315 320  
 His Ala Ala Ser Thr Gln Gly Gly Tyr Asp Met Arg Lys Leu Leu  
 325 330 335  
 Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser Val Thr Phe Val  
 340 345 350  
 Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val Gln  
 355 360 365  
 Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu Ser  
 370 375 380  
 Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly Asp  
 385 390 395 400  
 Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile Leu  
 405 410 415  
 Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe Asp  
 420 425 430  
 His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val Ala  
 435 440 445  
 Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Ala Lys  
 450 455 460  
 Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp Ile  
 465 470 475 480  
 Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp Gly  
 485 490 495  
 Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg

500

505

510

## (2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 520 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Met	Arg	Gly	Arg	Gly	Asn	Met	Ile	Gln	Lys	Arg	Lys	Arg	Thr	Val	Ser
1									10					15	
Phe	Arg	Leu	Val	Leu	Met	Cys	Thr	Leu	Leu	Phe	Val	Ser	Leu	Pro	Ile
								20	25				30		
Thr	Lys	Thr	Ser	Ala	Val	Asn	Gly	Thr	Leu	Met	Gln	Tyr	Phe	Glu	Trp
								35	40			45			
Tyr	Thr	Pro	Asn	Asp	Gly	Gln	His	Trp	Lys	Arg	Leu	Gln	Asn	Asp	Ala
								50	55			60			
Glu	His	Leu	Ser	Asp	Ile	Gly	Ile	Thr	Ala	Val	Trp	Ile	Pro	Pro	Ala
								65	70			75		80	
Tyr	Lys	Gly	Leu	Ser	Gln	Ser	Asp	Asn	Gly	Tyr	Gly	Pro	Tyr	Asp	Leu
								85	90			95			
Tyr	Asp	Leu	Gly	Glu	Phe	Gln	Gln	Lys	Gly	Thr	Val	Arg	Thr	Lys	Tyr
								100	105			110			
Gly	Thr	Lys	Ser	Glu	Leu	Gln	Asp	Ala	Ile	Gly	Ser	Leu	His	Ser	Arg
								115	120			125			
Asn	Val	Gln	Val	Tyr	Gly	Asp	Val	Val	Leu	Asn	His	Lys	Ala	Gly	Ala
								130	135			140			
Asp	Ala	Thr	Glu	Asp	Val	Thr	Ala	Val	Glu	Val	Asn	Pro	Ala	Asn	Arg
								145	150			155		160	
Asn	Gln	Glu	Thr	Ser	Glu	Glu	Tyr	Gln	Ile	Lys	Ala	Trp	Thr	Asp	Phe
								165	170			175			
Arg	Phe	Pro	Gly	Arg	Gly	Asn	Thr	Tyr	Ser	Asp	Phe	Lys	Trp	His	Trp
								180	185			190			
Tyr	His	Phe	Asp	Gly	Ala	Asp	Trp	Asp	Glu	Ser	Arg	Lys	Ile	Ser	Arg
								195	200			205			
Ile	Phe	Lys	Phe	Arg	Gly	Glu	Gly	Lys	Ala	Trp	Asp	Trp	Glu	Val	Ser
								210	215			220			
Ser	Glu	Asn	Gly	Asn	Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Val	Asp	Tyr
								225	230			235		240	
Asp	His	Pro	Asp	Val	Val	Ala	Glu	Thr	Lys	Lys	Trp	Gly	Ile	Trp	Tyr
								245	250			255			
Ala	Asn	Glu	Leu	Ser	Leu	Asp	Gly	Phe	Arg	Ile	Asp	Ala	Ala	Lys	His
								260	265			270			
Ile	Lys	Phe	Ser	Phe	Leu	Arg	Asp	Trp	Val	Gln	Ala	Val	Arg	Gln	Ala

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275	280	285
Thr Gly Lys Glu Met Phe Thr Val Ala Glu Tyr Trp Gln Asn Asn Ala		
290	295	300
Gly Lys Leu Glu Asn Tyr Leu Asn Lys Thr Ser Phe Asn Gln Ser Val		
305	310	315
320		
Phe Asp Val Pro Leu His Phe Asn Leu Gln Ala Ala Ser Ser Gln Gly		
325	330	335
Gly Gly Tyr Asp Met Arg Arg Leu Leu Asp Gly Thr Val Val Ser Arg		
340	345	350
His Pro Glu Lys Ala Val Thr Phe Val Glu Asn His Asp Thr Gln Pro		
355	360	365
Gly Gln Ser Leu Glu Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala		
370	375	380
Tyr Ala Phe Ile Leu Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr		
385	390	395
400		
Gly Asp Met Tyr Gly Thr Lys Gly Thr Ser Pro Lys Glu Ile Pro Ser		
405	410	415
Leu Lys Asp Asn Ile Glu Pro Ile Leu Lys Ala Arg Lys Glu Tyr Ala		
420	425	430
Tyr Gly Pro Gln His Asp Tyr Ile Asp His Pro Asp Val Ile Gly Trp		
435	440	445
Thr Arg Glu Gly Asp Ser Ser Ala Ala Lys Ser Gly Leu Ala Ala Leu		
450	455	460
Ile Thr Asp Gly Pro Gly Gly Ser Lys Arg Met Tyr Ala Gly Leu Lys		
465	470	475
480		
Asn Ala Gly Glu Thr Trp Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr		
485	490	495
Val Lys Ile Gly Ser Asp Gly Trp Gly Glu Phe His Val Asn Asp Gly		
500	505	510
Ser Val Ser Ile Tyr Val Gln Lys		
515	520	

## (2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 548 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Val	Leu	Thr	Phe	His	Arg	Ile	Ile	Arg	Lys	Gly	Trp	Met	Phe	Leu	Leu
1						5		10				15			
Ala Phe Leu Leu Thr Ala Ser Leu Phe Cys Pro Thr Gly Arg His Ala															
20 25 30															
Lys Ala Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp															

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35	40	45
Tyr Leu Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala		
50	55	60
Asn Asn Leu Ser Ser Leu Gly Ile Thr Ala Leu Ser Leu Pro Pro Ala		
65	70	75
Tyr Lys Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu		
85	90	95
Tyr Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr		
100	105	110
Gly Thr Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala		
115	120	125
Gly Met Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala		
130	135	140
Asp Gly Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg		
145	150	155
Asn Gln Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe		
165	170	175
Asp Phe Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp		
180	185	190
Tyr His Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg		
195	200	205
Ile Tyr Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp		
210	215	220
Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met		
225	230	235
Asp His Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr		
245	250	255
Val Asn Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Gly Leu Lys His		
260	265	270
Ile Lys Phe Ser Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln		
275	280	285
Thr Gly Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile		
290	295	300
Asn Lys Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu		
305	310	315
Phe Asp Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly		
325	330	335
Gly Ala Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp		
340	345	350
Gln Pro Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Asn Pro		
355	360	365
Ala Lys Arg Cys Ser His Gly Arg Pro Trp Phe Lys Pro Leu Ala Tyr		
370	375	380
Ala Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly		
385	390	395
		400

Asp Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys  
 405 410 415  
 Ile Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln  
 420 425 430  
 His Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly  
 435 440 445  
 Val Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly  
 450 455 460  
 Ala Gly Arg Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys  
 465 470 475 480  
 Val Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn  
 485 490 495  
 Ser Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val  
 500 505 510  
 Trp Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile Thr  
 515 520 525  
 Thr Arg Pro Trp Thr Gly Glu Phe Val Arg Trp His Glu Pro Arg Leu  
 530 535 540  
 Val Ala Trp Pro  
 545

## (2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 483 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro  
 1 5 10 15  
 Asn Asp Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu  
 20 25 30  
 Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly  
 35 40 45  
 Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu  
 50 55 60  
 Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys  
 65 70 75 80  
 Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn  
 85 90 95  
 Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr  
 100 105 110  
 Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val  
 115 120 125

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Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro  
 130 135 140  
 Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe  
 145 150 155 160  
 Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys  
 165 170 175  
 Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn  
 180 185 190  
 Tyr Asp Tyr Leu Thr Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val  
 195 200 205  
 Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln  
 210 215 220  
 Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe  
 225 230 235 240  
 Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met  
 245 250 255  
 Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn  
 260 265 270  
 Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu  
 275 280 285  
 His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Tyr Asp Met  
 290 295 300  
 Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser  
 305 310 315 320  
 Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu  
 325 330 335  
 Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu  
 340 345 350  
 Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly  
 355 360 365  
 Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile  
 370 375 380  
 Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His  
 385 390 395 400  
 Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp  
 405 410 415  
 Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro  
 420 425 430  
 Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr  
 435 440 445  
 Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser  
 450 455 460  
 Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr  
 465 470 475 480  
 Val Gln Arg

## (2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 487 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Ala Ala Ala Ala Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu  
1 5 10 15

Trp Tyr Met Pro Asn Asp Gly Gln His Trp Lys Arg Leu Gln Asn Asp  
20 25 30

Ser Ala Tyr Leu Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro  
35 40 45

Ala Tyr Lys Gly Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp  
50 55 60

Leu Tyr Asp Leu Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys  
65 70 75 80

Tyr Gly Thr Lys Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser  
85 90 95

Arg Asp Ile Asn Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly  
100 105 110

Ala Asp Ala Thr Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp  
115 120 125

Arg Asn Arg Val Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His  
130 135 140

Phe His Phe Pro Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His  
145 150 155 160

Trp Tyr His Phe Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn  
165 170 175

Arg Ile Tyr Lys Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn  
180 185 190

Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Tyr Asp  
195 200 205

His Pro Asp Val Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala  
210 215 220

Asn Glu Leu Gln Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile  
225 230 235 240

Lys Phe Ser Phe Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr  
245 250 255

Gly Lys Glu Met Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly  
260 265 270

Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe  
275 280 285

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Asp Val Pro Leu His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly  
 290 295 300  
 Gly Tyr Asp Met Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His  
 305 310 315 320  
 Pro Leu Lys Ser Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly  
 325 330 335  
 Gln Ser Leu Glu Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr  
 340 345 350  
 Ala Phe Ile Leu Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly  
 355 360 365  
 Asp Met Tyr Gly Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu  
 370 375 380  
 Lys His Lys Ile Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr  
 385 390 395 400  
 Gly Ala Gln His Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr  
 405 410 415  
 Arg Glu Gly Asp Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile  
 420 425 430  
 Thr Asp Gly Pro Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn  
 435 440 445  
 Ala Gly Glu Thr Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val  
 450 455 460  
 Val Ile Asn Ser Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser  
 465 470 475 480  
 Val Ser Ile Tyr Val Gln Arg  
 485

## (2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Met Lys Gln Gln Lys Arg Leu Thr Ala Arg Leu Leu Thr Leu Leu Phe  
 1 5 10 15  
 Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Asn Leu  
 20 25 30

## (2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Met Arg Ser Lys Thr Leu Trp Ile Ser Leu Leu Phe Ala Leu Thr Leu  
1 5 10 15

Ile Phe Thr Met Ala Phe Ser Asn Met Ser Ala Gln Ala Ala Gly Lys  
20 25 30

Ser

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Met Arg Ser Lys Thr Leu Trp Ile Ser Leu Leu Phe Ala Leu Thr Leu  
1 5 10 15

Ile Phe Thr Met Ala Phe Ser Asn Met Ser Ala Gln Ala Ala Ala Ala  
20 25 30

Ala Ala Asn  
35

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Met Arg Ser Lys Thr Leu Trp Ile Ser Leu Leu Phe Ala Leu Thr Leu  
1 5 10 15

Ile Phe Thr Met Ala Phe Ser Asn Met Ser Ala Gln Ala Ala Asn Leu  
20 25 30

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

CACCTAATTA AAGCTTCAC ACATTTCAT TTT 33

## (2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CACCTAATTA AAGCTTACAC ACATTTCAT TTT 33

## (2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 66 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CCGGCGTAATT TCCGGAGAAC ACCTAATTAA AGCCGCAACA CATTTCATT TTCCCGGGCG 60

CGGCAG 66

## (2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 42 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CCGGAGAACAC CCTAATTAAA GCCCTAACAC ATTTTCATTTC 42

## (2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 42 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CCGGAGAAC CCTAATTAAA GCCCACACAC ATTTTCATTT TC

42

(2) INFORMATION FOR SEQ ID NO:47:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 42 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CCGGAGAAC CCTAATTAAA GCCTGCACAC ATTTTCATTT TC

42

(2) INFORMATION FOR SEQ ID NO:48:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GATGCAGTAT TTCGAACCTGG TATA

24

(2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

TGCCCCAATGA TGGCCAACAT TGGAAG

26

(2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CGAATGGTAT GCTCCCAATG ACGG

24

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## (2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

CGAATGGTAT CGCCCCAATG ACGG

24

## (2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

CGAATGGTAT AATCCCCAATG ACGG

24

## (2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

CGAATGGTAT GATCCCCAATG ACGG

24

## (2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CGAATGGTAT CACCCCCAATG ACGG

24

## (2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CGAATGGTAT AAACCCAATG ACGG

24

(2) INFORMATION FOR SEQ ID NO:56:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CGAATGGTAT CCGCCCAATG ACGG

24

(2) INFORMATION FOR SEQ ID NO:57:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

CGAATGGTAT TCTCCCAATG ACGG

24

(2) INFORMATION FOR SEQ ID NO:58:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

CGAATGGTAC ACTCCCAATG ACGG

24

(2) INFORMATION FOR SEQ ID NO:59:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

CGAATGGTAT GTTCCAATG ACGG

24

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

CGAATGGTAT TGTCCAATG ACGG

24

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

CGAATGGTAT CAACCCAATG ACGG

24

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

CGAATGGTAT GAACCCAATG ACGG

24

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

CGAATGGTAT GGTCCCAATG ACGG

24

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

CGAATGGTAT ATTCCCAATG ACGG

24

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

CGAATGGTAT TTTCCCAATG ACGG

24

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

CGAATGGTAC TGGCCCAATG ACGG

24

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

CGAATGGTAT TATCCCAATG ACGG

24

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(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

CCGTCATTGG GACTACGTAC CATT

24

**WHAT IS CLAIMED IS:**

1. An improved laundry detergent composition, the improvement comprising adding to the laundry detergent composition a mutant alpha-amylase that is the expression product of a mutated DNA sequence encoding an alpha-amylase, the mutated DNA sequence being derived from a precursor alpha-amylase by the substitution of a methionine at a position equivalent to M+197 in *B. licheniformis* alpha-amylase and the substitution of one or more methionine or tryptophan at a position equivalent to M+15 or W+138 in *B. licheniformis* alpha-amylase.
2. An improved laundry detergent composition of Claim 1 wherein the cleaning composition is a liquid composition.
3. An improved laundry detergent composition of Claim 1 wherein the mutant alpha-amylase is selected from the group consisting of M15T/M197T; M15S/M197T; W138Y/M197T; M15S/W138Y/M197T and M15T/W138Y/M197T.
4. An improved laundry detergent composition of Claim 1 further comprising a mutant protease that is the expression product of a mutated DNA sequence encoding a protease, the mutated DNA sequence being derived from a precursor protease by the substitution of a methionine at a position equivalent to M+222 in *Bacillus amyloliquefaciens* protease.
5. An improved laundry detergent composition of Claim 4 wherein the mutant protease comprises a substitution selected from the group of amino acids consisting of alanine, cysteine and serine.
6. An improved laundry detergent composition of Claim 4 comprising an alpha-amylase mutant selected from the group consisting of M15T/M197T, M15S/M197T, W138Y/M197T, M15S/W138Y/M197T and M15T/W138Y/M197T, and a protease mutant selected from the group consisting of M222C, M222S and M222A.

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7. An improved laundry detergent composition of Claim 2, wherein the liquid detergent is at a pH of between about 6.0 and 10.0.
8. An improved laundry detergent composition according to claim 1, wherein said laundry detergent contains bleach.
9. An improved laundry detergent composition according to claim 8, wherein said laundry detergent has a pH of above about 10.
10. An improved laundry detergent composition according to claim 9, wherein said laundry detergent is granular.

10 30 50  
 AGCTTGAAGAAGTGAAGAAGCAGAGAGGCTATTGAATAATGAGTAGAAAGCGCCATATC  
 70 90 110  
 GGCGCTTTCTTTGGAAGAAAATAGGGAAAATGGTACTTGTAAAAATTGGAATAT  
 130 150 170  
 TTATACAACATCATATGTTCACATTGAAAGGGAGGAGAACATGAAACAACAAAACG  
 M K Q Q K R  
 190 210 230  
 GCTTTACGCCCGATTGCTGACGCTGTTATTGCGCTCATCTTCTTGCCTGCCTCATTCTGC  
 L Y A R L L T L L F A L I F L L P H S A  
 250 270 290  
 AGCAGCGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTGAATGGTACATGCCAA  
 A A A A N L N G T L M Q Y F E W Y M P N  
 310 330 350  
 TGACGGCCAACATTGGAAGCGTTGCAAAACGACTCGGCATATTGGCTGAACACGGTAT  
 D G Q H W K R L Q N D S A Y L A E H G I  
 370 390 410  
 TACTGCCGCTGGATTCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGCTACGG  
 T A V W I P P A Y K G T S Q A D V G Y G  
 430 450 470  
 TGCTTACGACCTTATGATTAGGGAGTTCATCAAAAGGGACGGTTCGGACAAAGTA  
 A Y D L Y D L G E F H Q K G T V R T K Y  
 490 510 530  
 CGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGT  
 G T K G E L Q S A I K S L H S R D I N V  
 550 570 590  
 TTACGGGGATGGTCATCAACCACAAAGGCGGGCTGATGCGACCGAAGATGTAACCGC  
 Y G D V V I N H K G G A D A T E D V T A  
 610 630 650  
 GGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTCAAGGAGAACACCTAATTAAAGC  
 V E V D P A D R N R V I S G E H L I K A  
 670 690 710  
 CTGGACACATTTCATTTCGGGGCGGGCAGCACATACAGCGATTAAATGGCATTG  
 W T H F H F P G R G S T Y S D F K W H W  
 730 750 770  
 GTACCATTTGACGGAACCGATTGGGACGGAGTCCCGAAAGCTGAACCGCATCTATAAGTT  
 Y H F D G T D W D E S R K L N R I Y K F  
 790 810 830  
 TCAAGGAAAGGCTTGGGATTGGGAAGTTCCAATGAAAACGGCAACTATGATTATTGAT  
 Q G K A W D W E V S N E N G N Y D Y L M

**FIG. - 1A**

850	870	890
GTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAAATTAAAGAGATGGGGCAC		
Y A D I D Y D H P D V A A E I K R W G T		
910	930	950
TTGGTATGCCAATGAACTGCAATTGGACGGTTCCGTCTTGATGCTGTCAAACACATTAA		
W Y A N E L Q L D G F R L D A V K H I K		
970	990	1010
ATTTCTTTTGCAGGGATTGGGTTAACATGTCAGGGAAAAACGGGGAGGAAATGTT		
F S F L R D W V N H V R E K T G K E M F		
1030	1050	1070
TACGGTAGCTGAATATTGGCAGAATGACTGGCGCTCTGGAAAATTTGAACAAAAC		
T V A E Y W Q N D L G A L E N Y L N K T		
1090	1110	1130
AAATTTAACATTCAAGTGTGACGTGCCGCTTCATTATCAGTCCATGCTGCATCGAC		
N F N H S V F D V P L H Y Q F H A A S T		
1150	1170	1190
ACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGTTCCAAGCATCC		
Q G G G Y D M R K L L N G T V V S K H P		
1210	1230	1250
GTTGAAATCGGTTACATTTGTCGATAACCATGATAACAGCCGGGCAATCGCTTGAGTC		
L K S V T F V D N H D T Q P G Q S L E S		
1270	1290	1310
GAAGTCCAAACATGGTTAACGGCTTGCTTACGCTTTATTCTCACAGGGAAATCTGG		
T V Q T W F K P L A Y A F I L T R E S G		
1330	1350	1370
ATACCCCTCAGGTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAAT		
Y P Q V F Y G D M Y G T K G D S Q R E I		
1390	1410	1430
TCCTGCCCTGAAACACAAAATTGAACCGATCTAAACCGCAGAAACAGTATGCGTACGG		
P A L K H K I E P I L K A R K Q Y A Y G		
1450	1470	1490
AGCACAGCATGATTATTCGACCACCATGACATTGTCGGCTGGACAAGGGAGGCGACAG		
A Q H D Y F D H H D I V G W T R E G D S		
1510	1530	1550
CTCGGTTGCAAATTCAAGTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCG		
S V A N S G L A A L I T D G P G G A K R		
1570	1590	1610
AATGTATGTCGGCCGGCAAAACGCCGTGAGACATGGCATGACATTACCGGAAACCGTT		
M Y V G R Q N A G E T W H D I T G N R S		
1630	1650	1670
GGAGCCGGTTGTCAATTGGAGGCTGGGAGAGTTACGTAAACGGCGGGTCGGT		
E P V V I N S E G W G E F H V N G G S V		

**FIG.\_1B**

1690 1710 1730  
TTCAATTATGTTCAAAGATAGAAGAGCAGAGAGGACGGATTCCTGAAGGAAATCCGTT  
S I Y V Q R \*

1750 1770 1790  
TTTTATTTGCCCGTCTTATAAATTCTTGATTACATTTATAATTAAATTAAACAAA

1810 1830 1850  
GTGTCATCAGCCCTCAGGAAGGACTTGCTGACAGTTGAATCGCATAGGTAAGGCAGGGGA

1870 1890 1910  
TGAAATGGCAACGTTATCTGATGTAGCAAAGAAAGCAAATGTGTCAAAATGACGGTATC

1930 1950  
CGGGGTGATCAATCATCCTGAGACTGTGACGGATGAATTGAAAAAGCT

## ***FIG.\_1C***

***FIG.\_1A***

***FIG.\_1B***

***FIG.\_1C***

***FIG.\_1***

10 30 50  
ANLNQTLIMQYFEWYMPNDGQHWKRLQND SAY LAEH GIT AVWIP PAY KGT SQAD VGY GAYD

70 90 110  
LYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEV

130 150 170  
DPADRN RVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGK

190 210 230  
AWDWEVSNENGNYDYLMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLD AVKHIKFSF

250 270 290  
LRDWVNHVREKTGKEMFTVAEYWQNDLGALEN YLNKTNF NH SVFDVPLHYQFHAA STQGG

310 330 350  
GYDMRKLLNGTVVSKHPLKS VTFVDN HDT QPGQS LESTVQTWF KPLAYA FILTRES GYPQ

370 390 410  
VFYGD MYG TKG DSQ REIP AL KH KIEP IL KARK QYAY GAQHDY FDH DIVG WT REGD SSVA

430 450 470  
NSGLAALITDGPGGAKR MYVGRQNA GETWHD ITGNR SEP VV INSEG WGEFHVNGGSV SIY

VQR

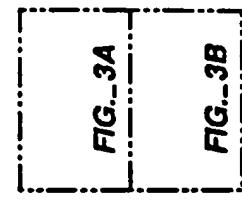
**FIG.\_2**

Am-Lich = <i>B. Licheniformis</i>	Am-Amylo = <i>B. amyloliquefaciens</i>	Am-Searo = <i>B. stearothiomophilus</i>
1	MKQQ KRYARLLTL LFALIFLLPH LMCTLLFVSL LLAFLLTASL	SAAA AANLNGLTLMQ TSAVNGTLMQ AAPFNGTMMQ
Am-Lich	MKQQ KRYARLLTL LFALIFLLPH LMCTLLFVSL LLAFLLTASL	SAAA AANLNGLTLMQ TSAVNGTLMQ AAPFNGTMMQ
Am-Amylo	MGRGQNMHQK RKTYSFRLV VLT	FCPTGRHAKA
Am-Searo	TLWTKVANE	
61	QHWKRLQND S AYLAEHGITA VWIPPAYKGT VWIPPAYKGL VSLPPAYKGL	DLYDLGEFHQ DLYDLGEFQQ DLYDLGEFNQ
Am-Lich	QHWKRLQND S AYLAEHGITA VWIPPAYKGL VSLPPAYKGL	DLYDLGEFHQ DLYDLGEFQQ DLYDLGEFNQ
Am-Amylo	QHWKRLQND S AYLAEHGITA VWIPPAYKGL VSLPPAYKGL	DLYDLGEFHQ DLYDLGEFQQ DLYDLGEFNQ
Am-Searo	TLWTKVANE	
121	KGELOSAIKS LHSRDINVYG DVVINKGGA DATEDVTAVE VDPADRNRI	DATEDVTAVE VNPANRNQET VNPSDRNQEI
Am-Lich	KSELQDAIGS LHSRNQVYVG DVVLNHKAGA DATEDVTAVE VDPADRNRI	DATEDVTAVE VNPANRNQET VNPSDRNQEI
Am-Amylo	KAQYLQAIQA AHAAGMQVYA DVVFDDHKGGA DGTEWWDAGE	DATEDVTAVE VNPANRNQET VNPSDRNQEI
Am-Searo		
181	HFHFPGRGST YSDFKWHHWYH FDGTDWDESR KLNRIYKF...	QGKAWDWEVS EKGAWDWEVS IGKAWDWEVD
Am-Lich	DFRFPGRGNT YSDFKWHHWYH FDGADWDESR KISRIFKFRG	NENGNYDYL M
Am-Amylo	KFDFFPGRGNT YSSFKWRRWYH FDGVWDWDESR KLSRIYKFRRG	SENGNYDYL M
Am-Searo		TENGNYDYL M
241	YADIDYDHPD VAAEIKRWGT WYANELQLDG FRDAWKHK	FSFLRDWVNH VREKTGKEMF
Am-Lich	YADVDYDHPD VVAETKWKGI WYANELSDLG FRDAAKHK	FSFLRDWVQA VROATGKEMF
Am-Amylo	YADLDMHPE VVTELKNWKGK WYVNNTNDG <u>FRLDGLKH</u>	FSFFPDWLSY VRSQTGKPL
Am-Searo		
301	TVAEYWQNDL GALENYLNKT NFNHSVFDVP LHYQFHAAST QGGGYDMRKL	317
Am-Lich	TVAEYWQNDL GALENYLNKT SFNQSVFDVP LHFNLQAAASS QGGGYDMRRL	360
Am-Amylo	TVAEYWQNDL GALENYLNKT NGTMSLFDAP LHNKFYTASK SGGAFDMRTL	
Am-Searo	TVGEYWWSYDI NKLHNYITKT	

**FIG.-3A**

361	Am-Lich Am-Amylo Am-Stearo	LKSVTFFVDNH EKAVTFVENH TLAVTFVDNH	DTQPGOSLES DTQPGOSLES <u>DTNPAKR</u> <u>DTNPAKR..CS</u>	TVQTWFKPLA TVQTWFKPLA HGRPWFKPLA	YAFILTRESG YAFILTRESG YAFILTRESG	YPOQVFYGDMDY YPOQVFYGDMDY YPCVFYGDYY	GTKGDSQREI GTKGTSPEI GI.....PQYNI	377 420
421	Am-Lich Am-Amylo Am-Stearo	PALKHKIEPI PSLKDNIEPI PSLKSKEIDPL	LKARKQYAYG LKARKEYAYG LIARRDYAYG	AQHDYFDHHD PQHDIIDHDP TQHDIYLDHSD	IVGWTREGDS VIGWTREGDS IIGWTREGVT	SVANSGLAAL SAAKSGGLAAL EKPGSGLAAL	ITDGPGGAKR ITDGPGGSKR ITDGAGRSKW	437 480
481	Am-Lich Am-Amylo Am-Stearo	MYVGRONAGE MYAGLKNAGE MYVKGGHAGK	TWHDITGNRS TWDITGNRS VFYDLTGNRS	EPVVINSEGW DTVKIGSDGW DTVTINSDGW	GEFHVNNGSV GEFHVNNGSV GEFKVNNGSV	SIYVQR..... SIYVQK..... SVWWPRKTTV	..... ..... STIARPITR	483 540
541	Am-Lich Am-Amylo Am-Stearo	..... PWTGEFVRWH .....	..... EPRLVAWP	559	.....	.....	.....	

**FIG. - 3B**



**FIG. - 3**

**FIG. 3B**

10 30 50  
ANLNGLTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYD

70 90 110  
LYDLGEFHQKGTVRTKYGTKGELOSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEV

130 150 170  
DPADRNVRVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRLNRIYKFQGK

190 210 230  
AWDWEVSNENGNYDYLTYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSF

250 270 290  
LRDWVNHVREKTGKEMFTVAEYWQNDLGALENYLNKTNFNSVFDVPLHYQFHAASTQGG

310 330 350  
GYDMRKLLNGTVVSKHPLKSFTVDNHDTPQPGQSLESTVQTWFKPLAYAFILTRESGYPQ

370 390 410  
VFYGDMDYGTKGDSQREIPALKHIEPILKARKQYAYGAQHDYFDHHDIVGWTREGDSSVA

430 450 470  
NSGLAALITDGPAGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHNGGSVSIY

VQR

**FIG.\_4a**

AAAA

14 34 54  
ANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKTSQADVGYGYAD

74 94 114  
LYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEV

134 154 174  
DPADRNVRVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGK

194 214 234  
AWDWEVSNENGNYDYLMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSF

254 274 294  
LRDWVNHVREKTGKEMFTVAEYWQNDLGALENYLNKTNFNSVFDVPLHYQFHAASTQGG

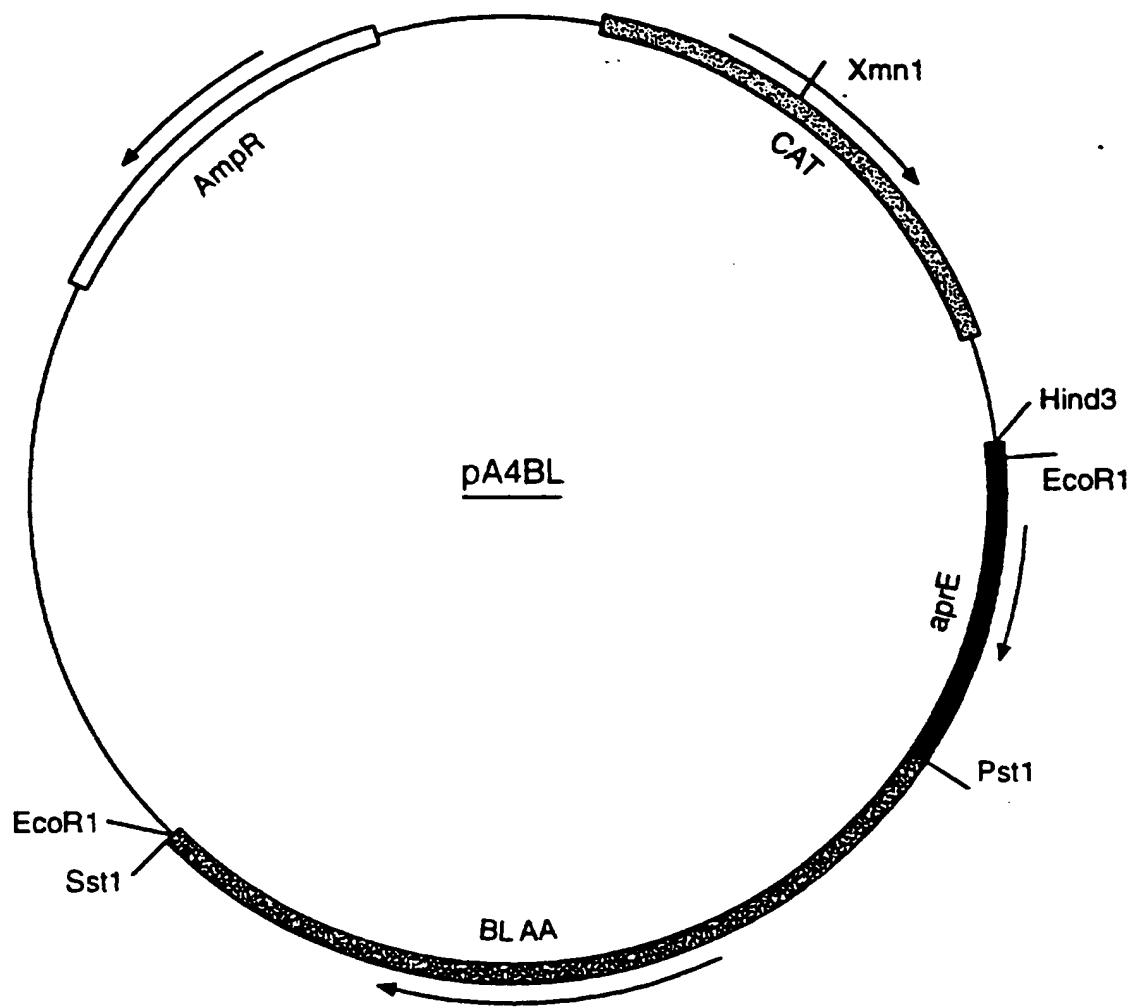
314 334 354  
GYDMRKLLNGTVVSKHPLKSVTFVDNHDTQPGQSLESTVQTWFKPLAYAFILTRESGYPQ

374 394 414  
VFYGDMDYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWTREGDSSVA

434 454 474  
NSGLAALITDGPGGAKRMYVGRONAGETWHIDITGNRSEPVVINSEGWGEFHNGGSVSIY

VQR

***FIG.\_4b***



**FIG. 5**

SIGNAL SEQUENCE - MATURE PROTEIN JUNCTIONS IN:*B.licheniformis* alpha-amylase.

M K Q Q K R L T A R L L T L L F A L I F L L P H S A A A A [ A N L .....  
(PstI) ↓  
N-terminus

*B. subtilis* alkaline protease aprE.

M R S K T L W I S L L F A L T L I F T M A F S N M S A Q A [ A G K S .....  
(PstI) ↓  
N-terminus

*B.licheniformis* alpha-amylase in pA4BL.

M R S K T L W I S L L F A L T L I F T M A F S N M S A Q A [ A A A A A N .  
(PstI) ↓  
N-terminus

*B.licheniformis* alpha-amylase in pBLapr.

M R S K T L W I S L L F A L T L I F T M A F S N M S A Q A [ A N L .....  
N-terminus

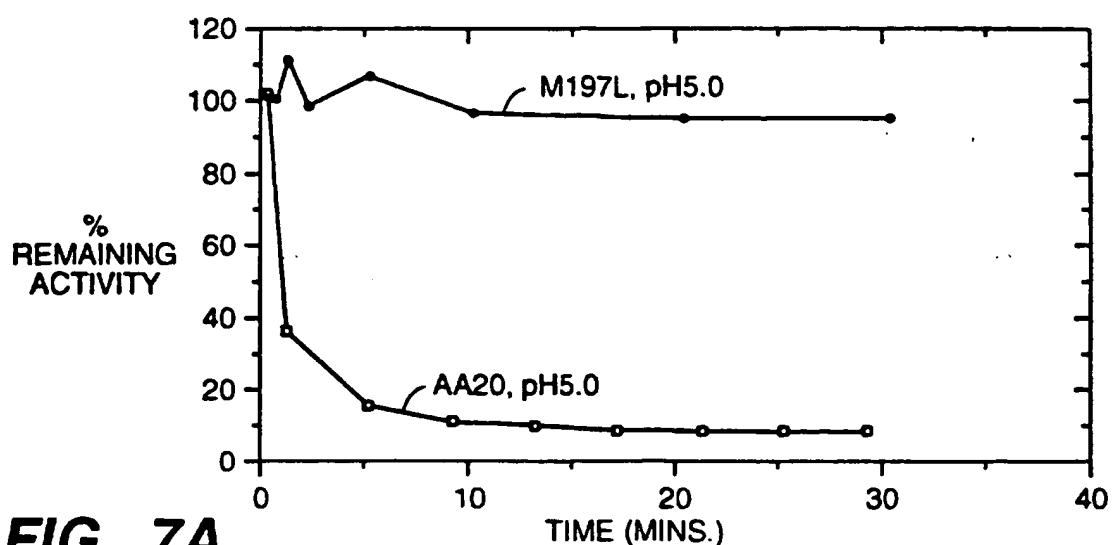
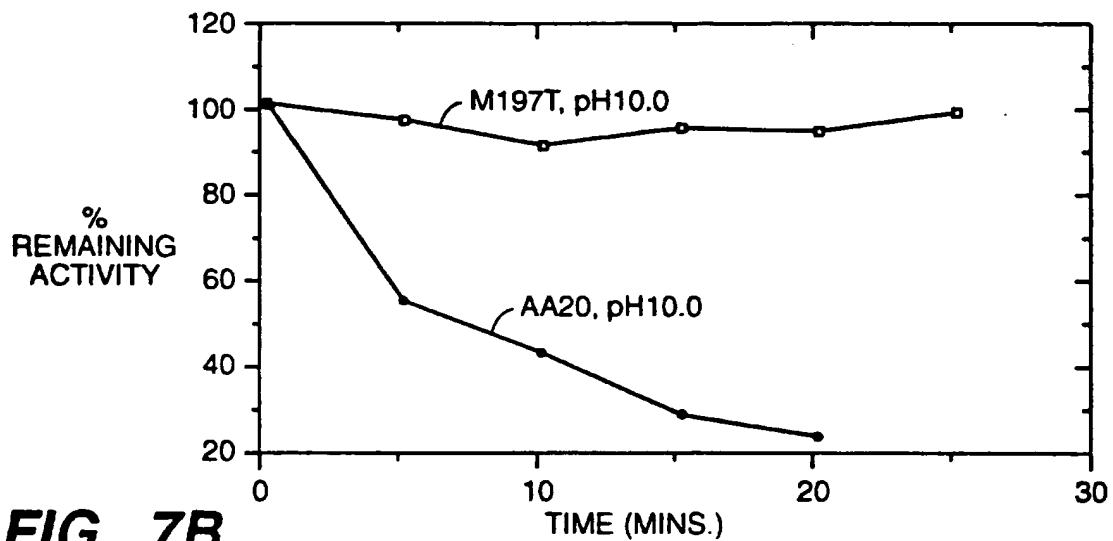
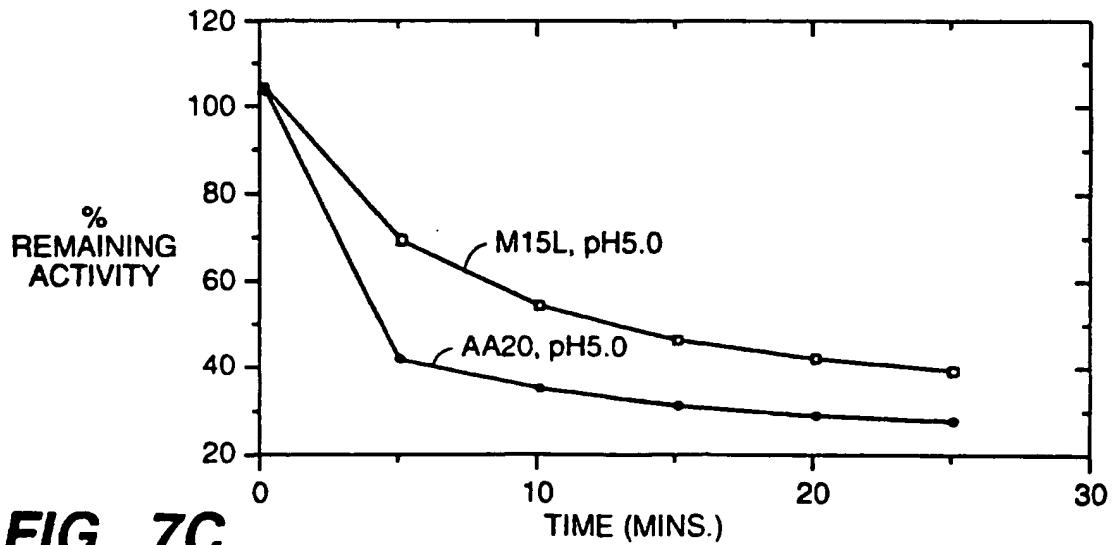
(PstI) indicates the site of the restriction site in the gene.



[ ] indicates cleavage site between signal peptide and secreted protein.

**FIG.\_6**

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**FIG.\_7A****FIG.\_7B****FIG.\_7C**

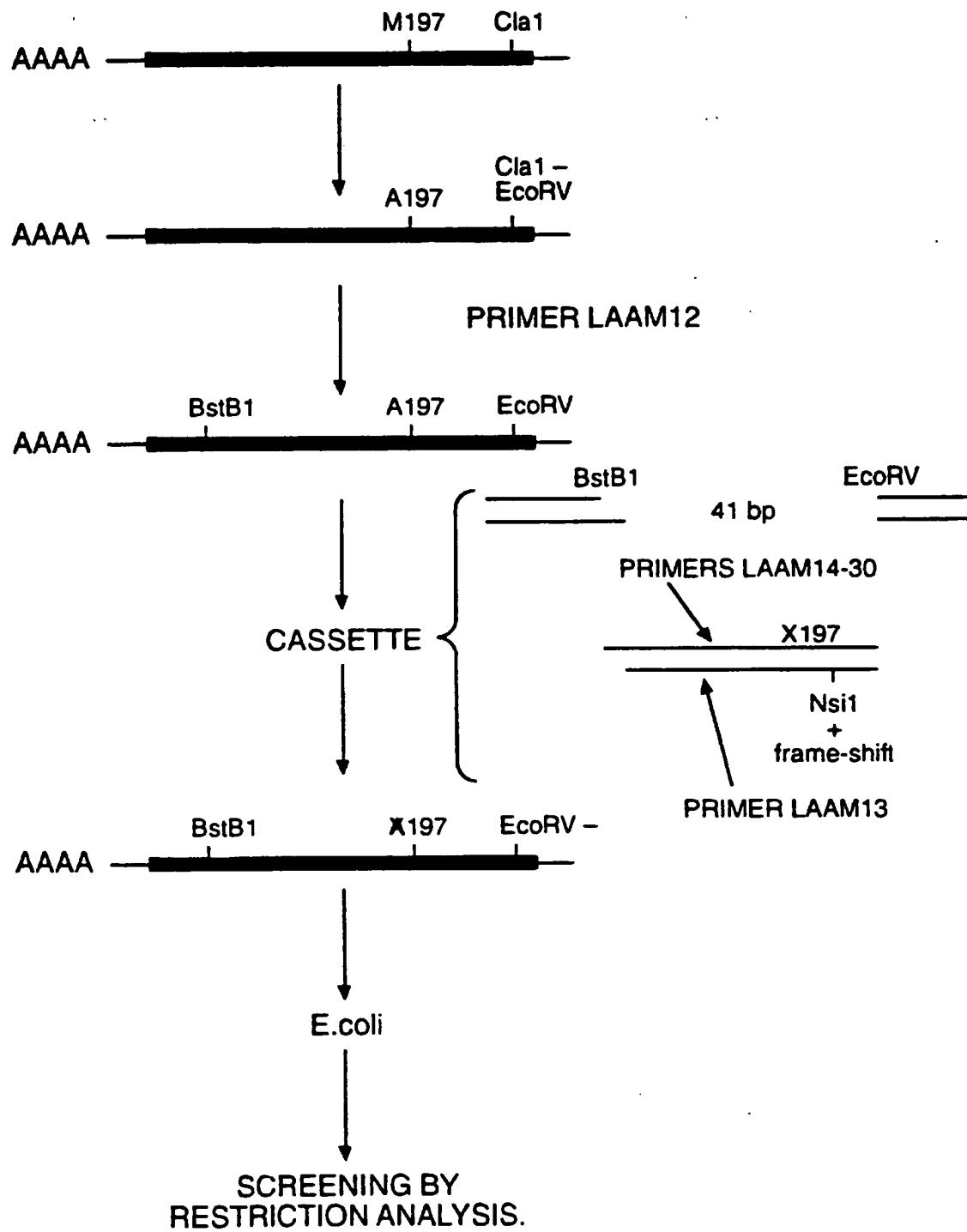


FIG.\_8

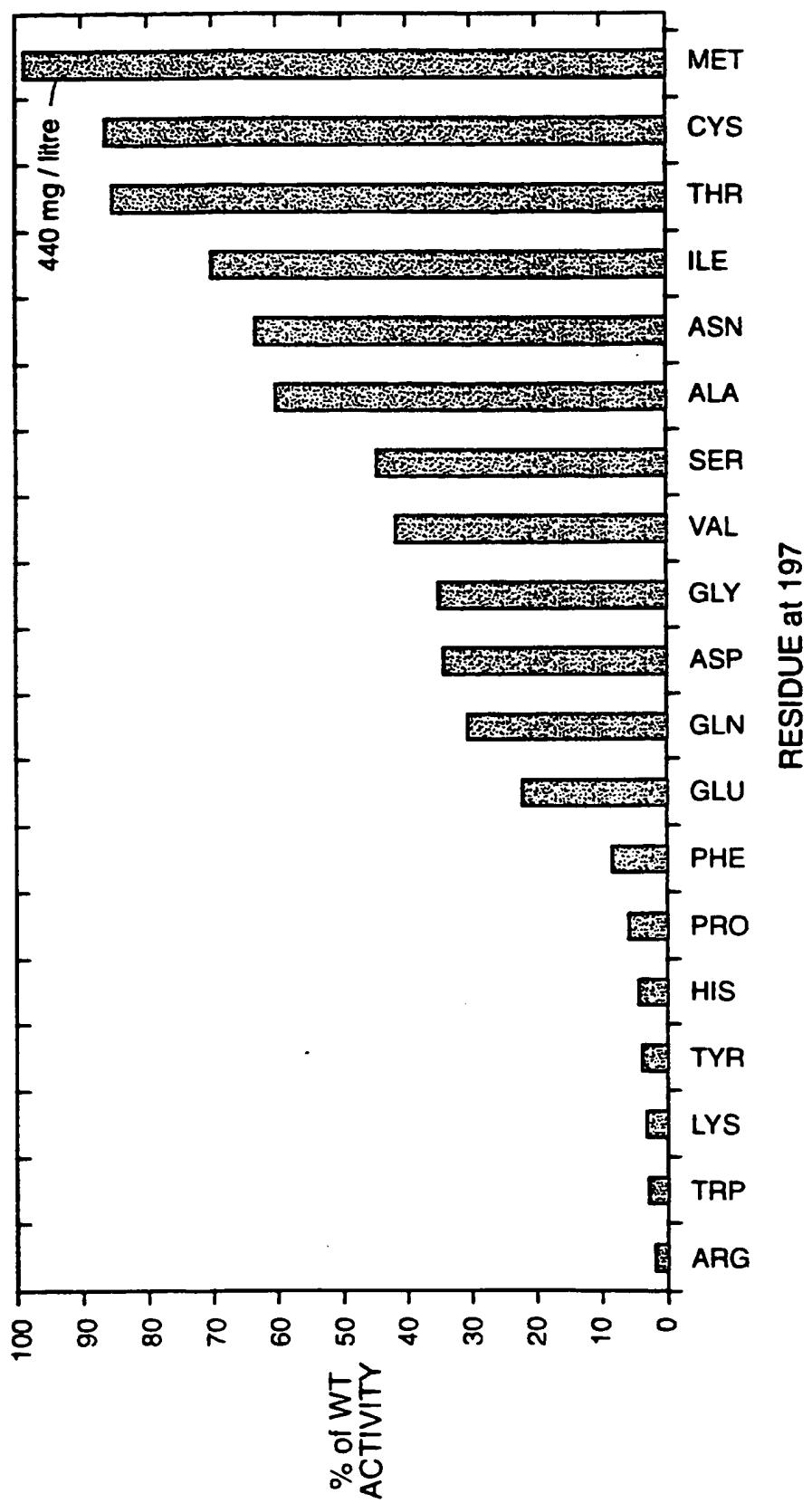


FIG. 9

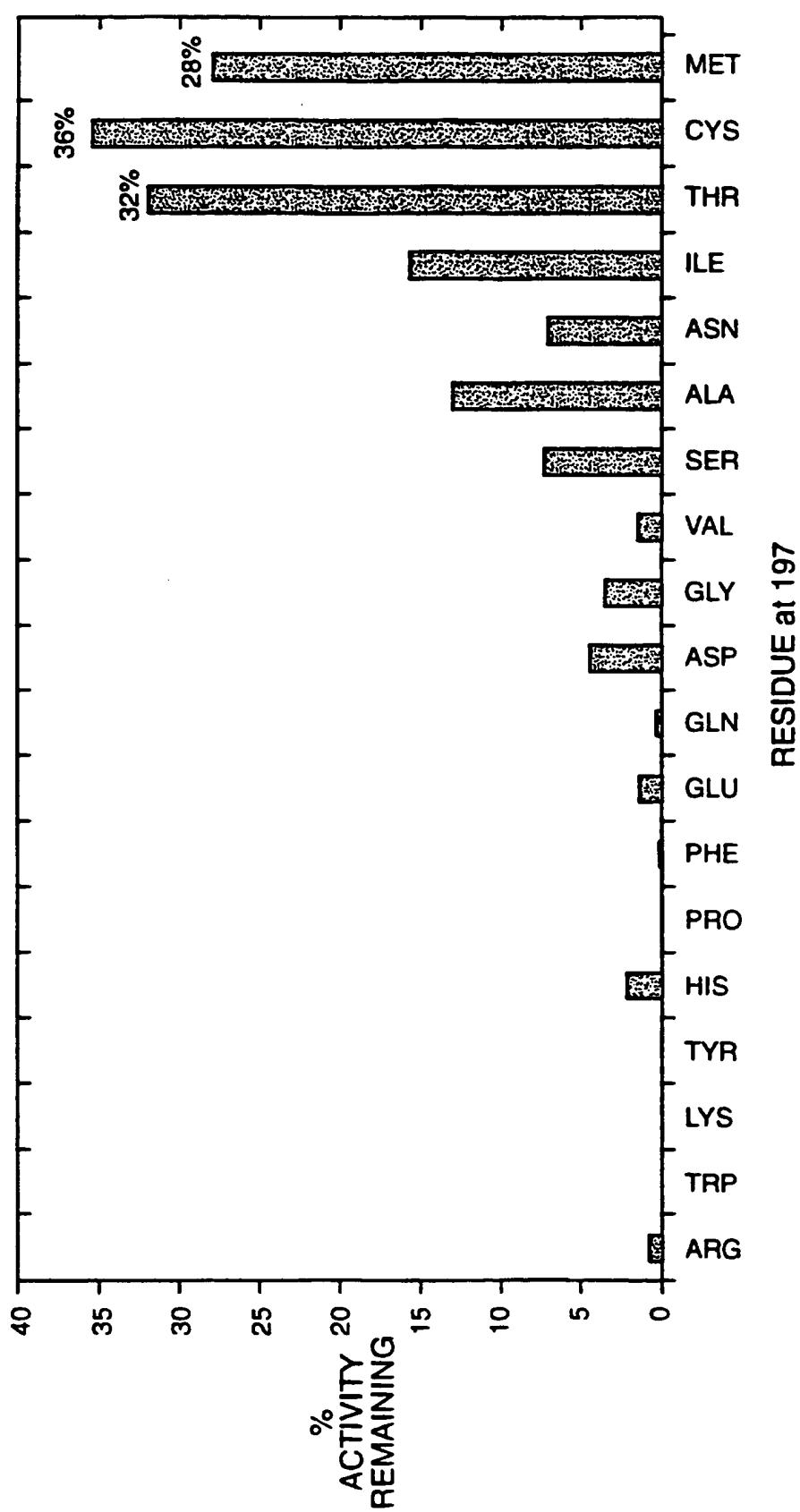
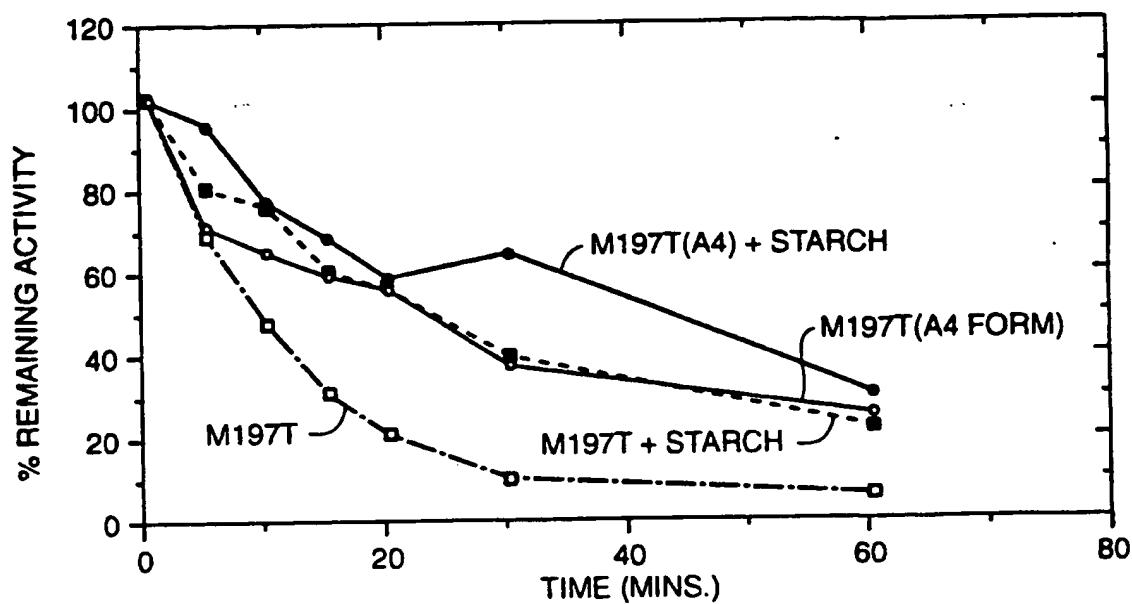
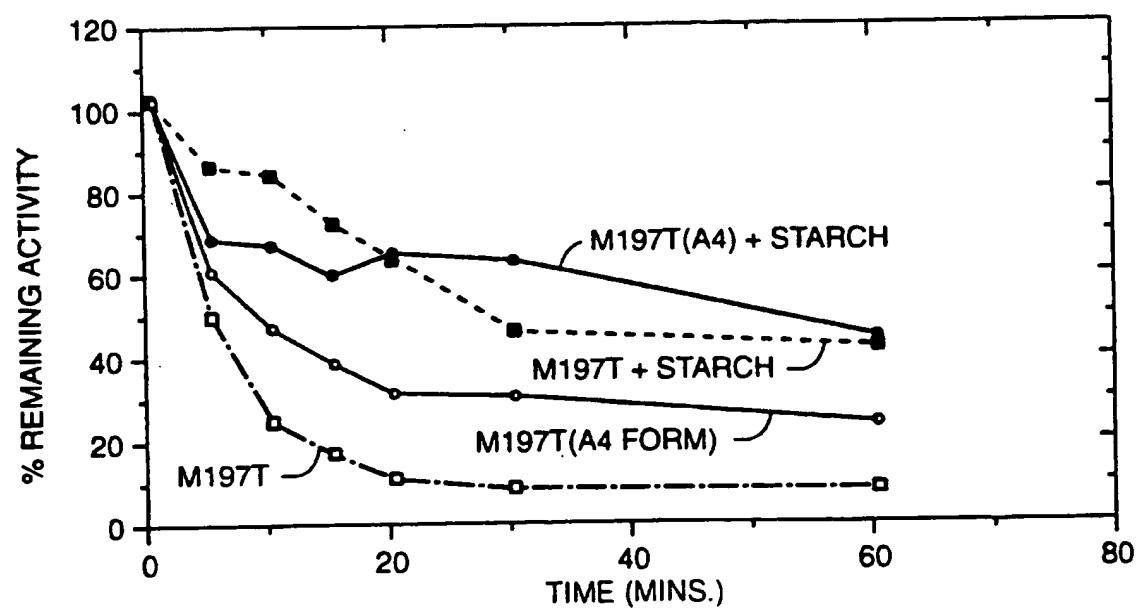
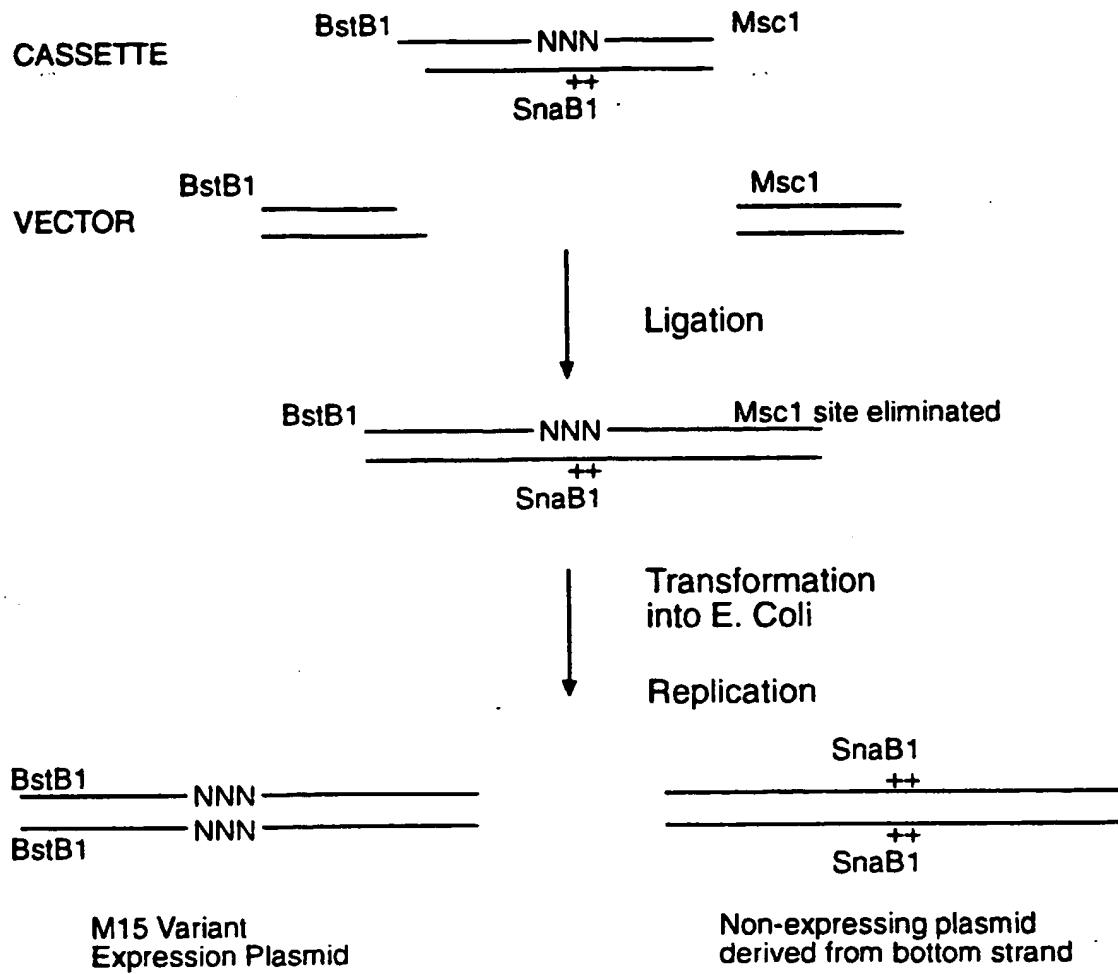


FIG. 10

**FIG.\_11A****FIG.\_11B**

**FIG. 12**

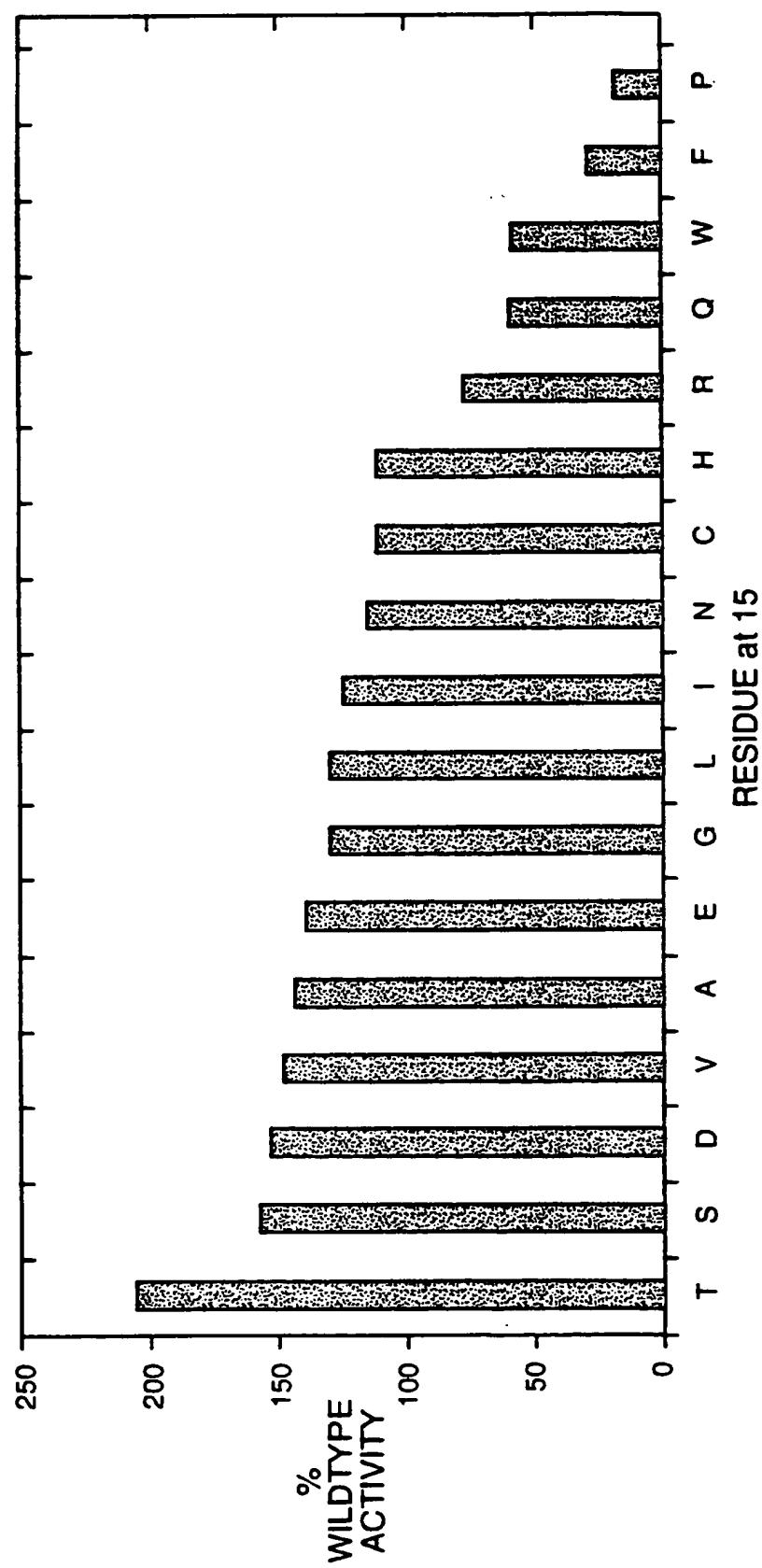


FIG. 13

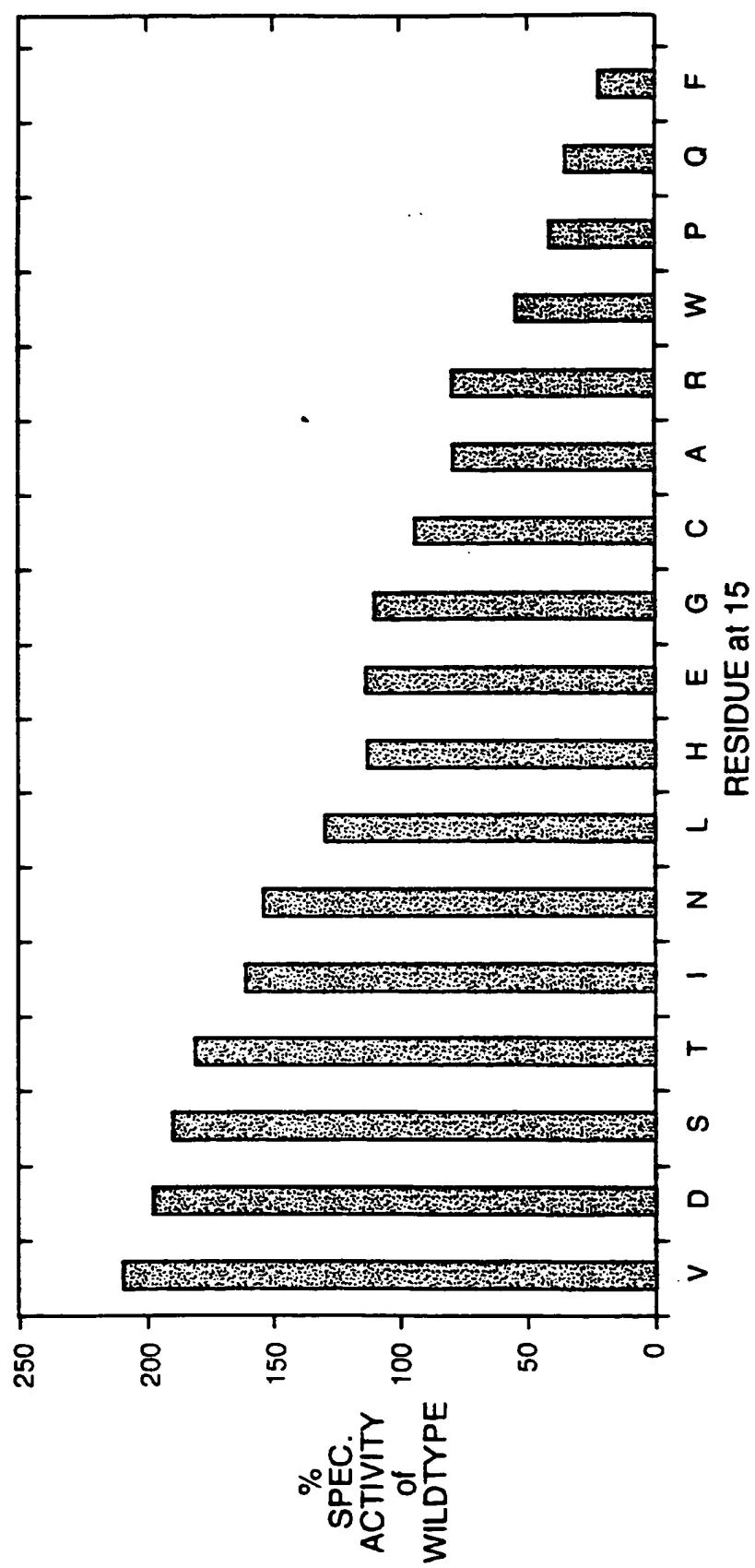


FIG. 14

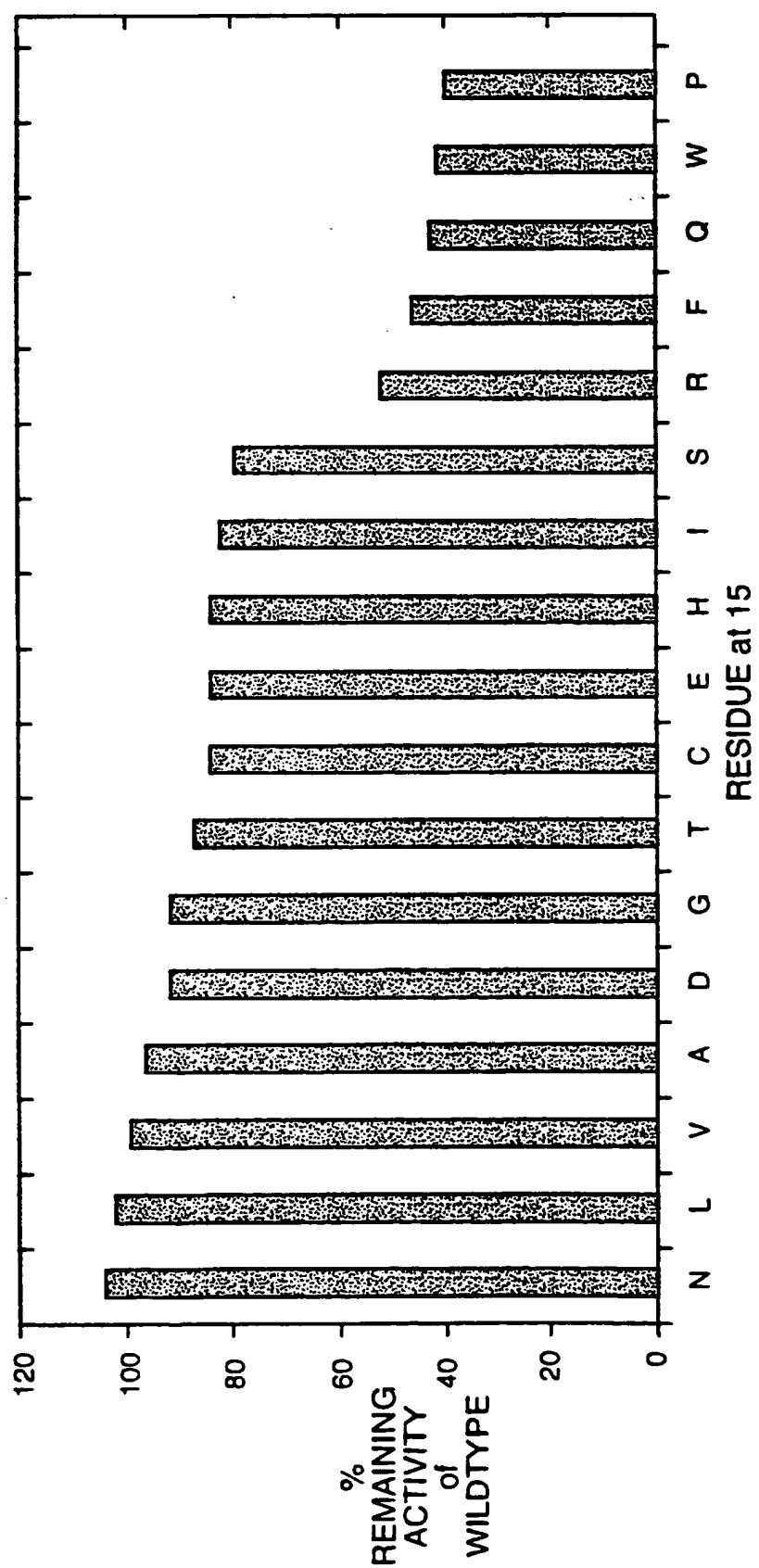


FIG. 15

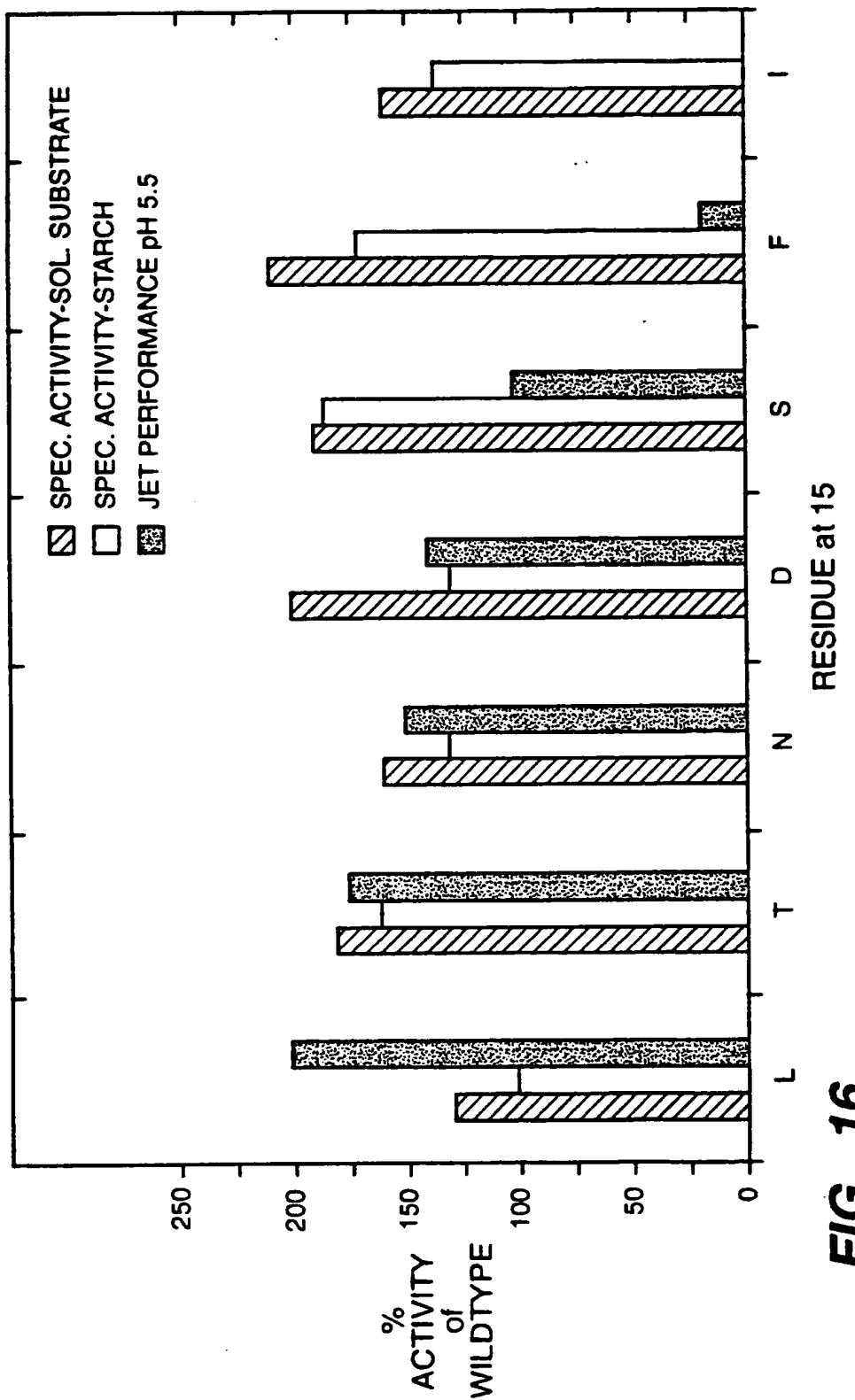
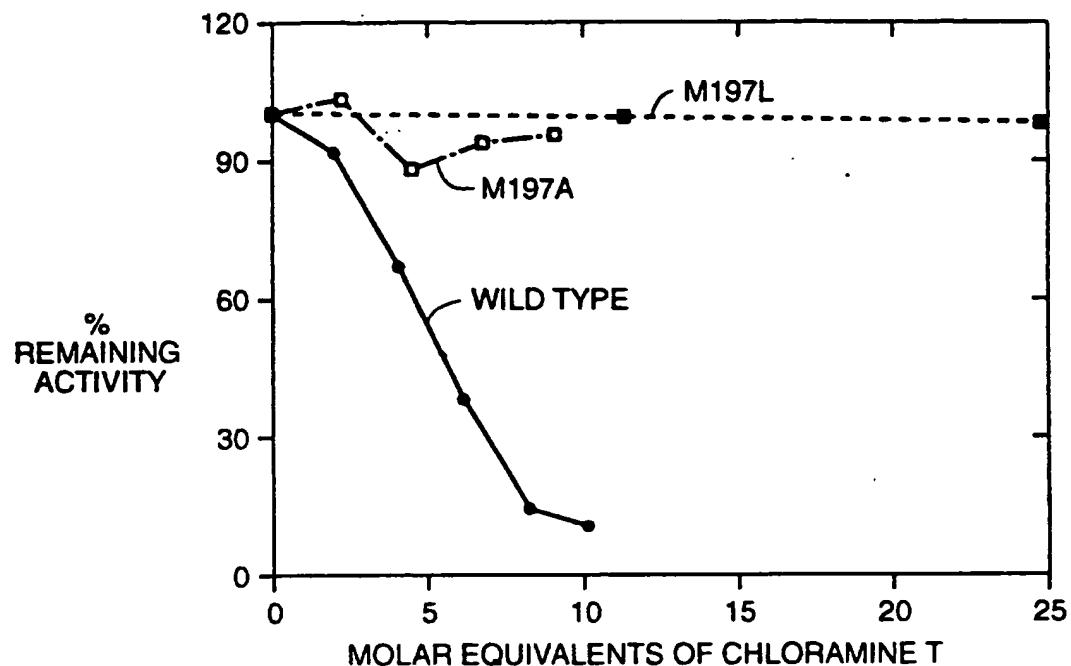
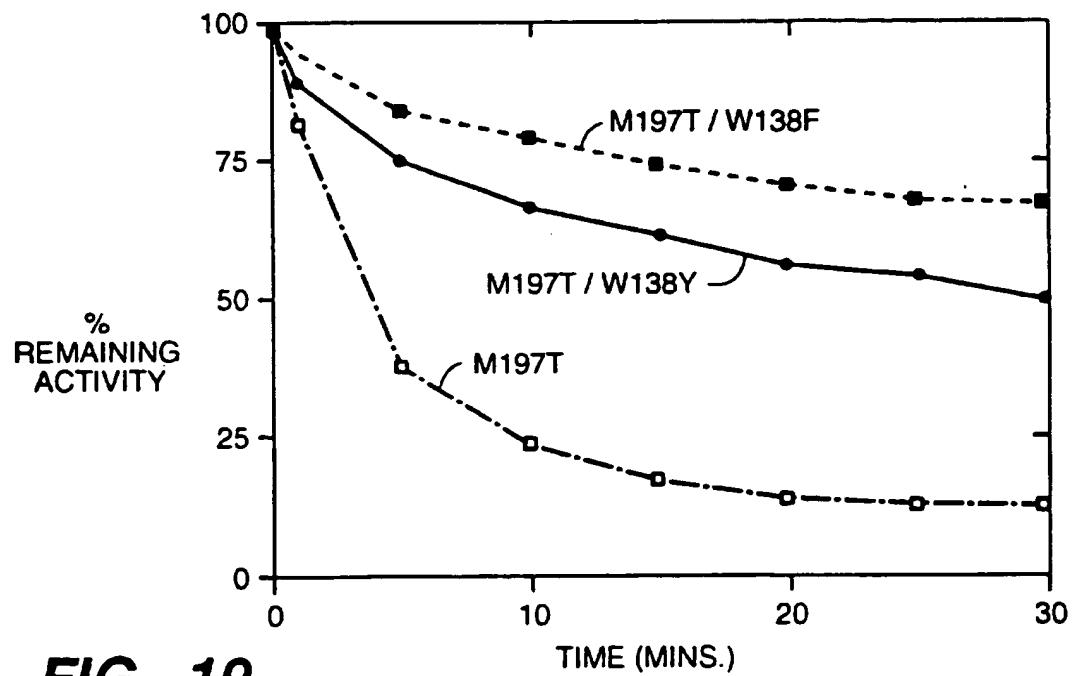


FIG. 16

**FIG.\_17****FIG.\_19**

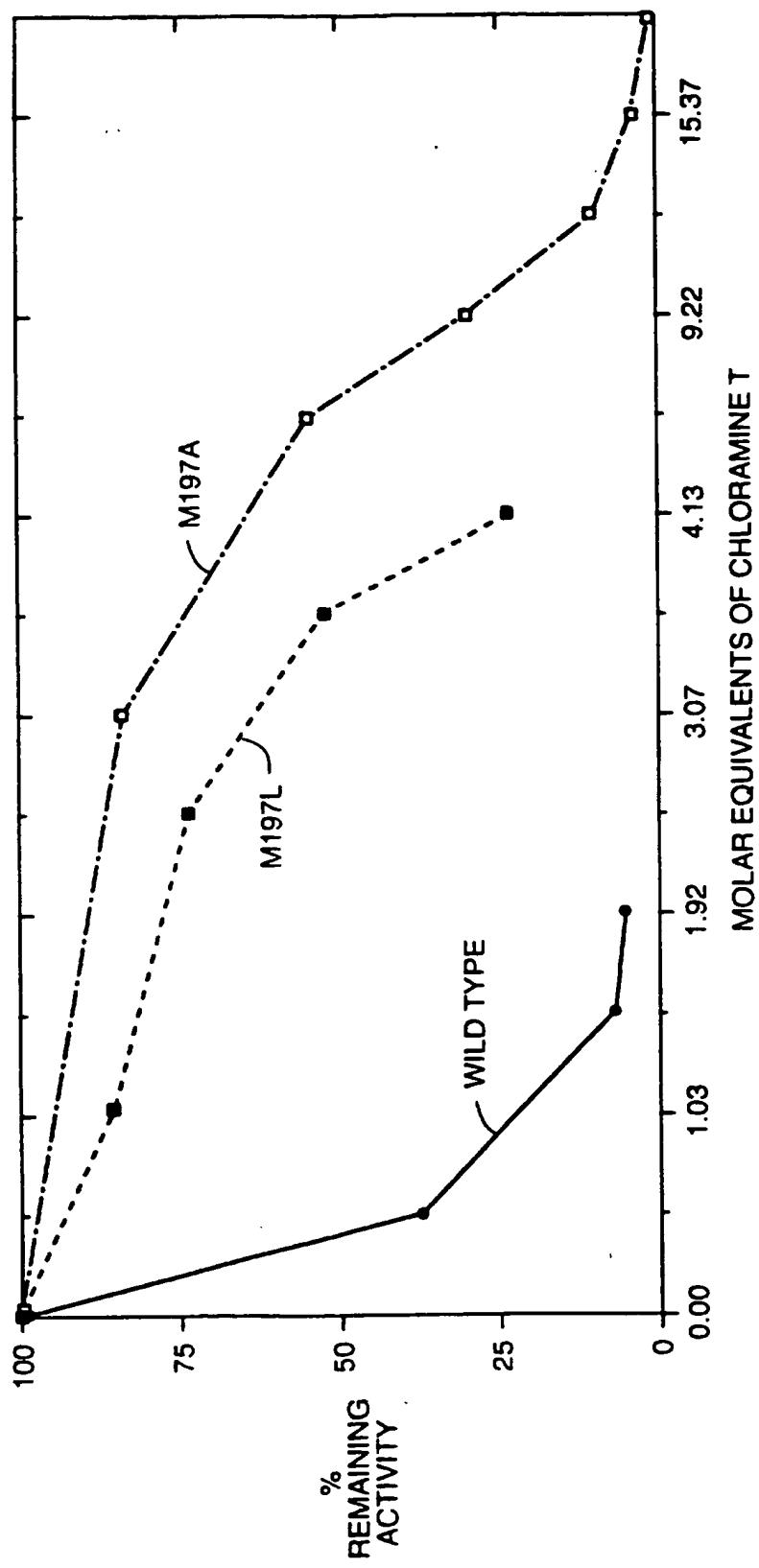


FIG. 18

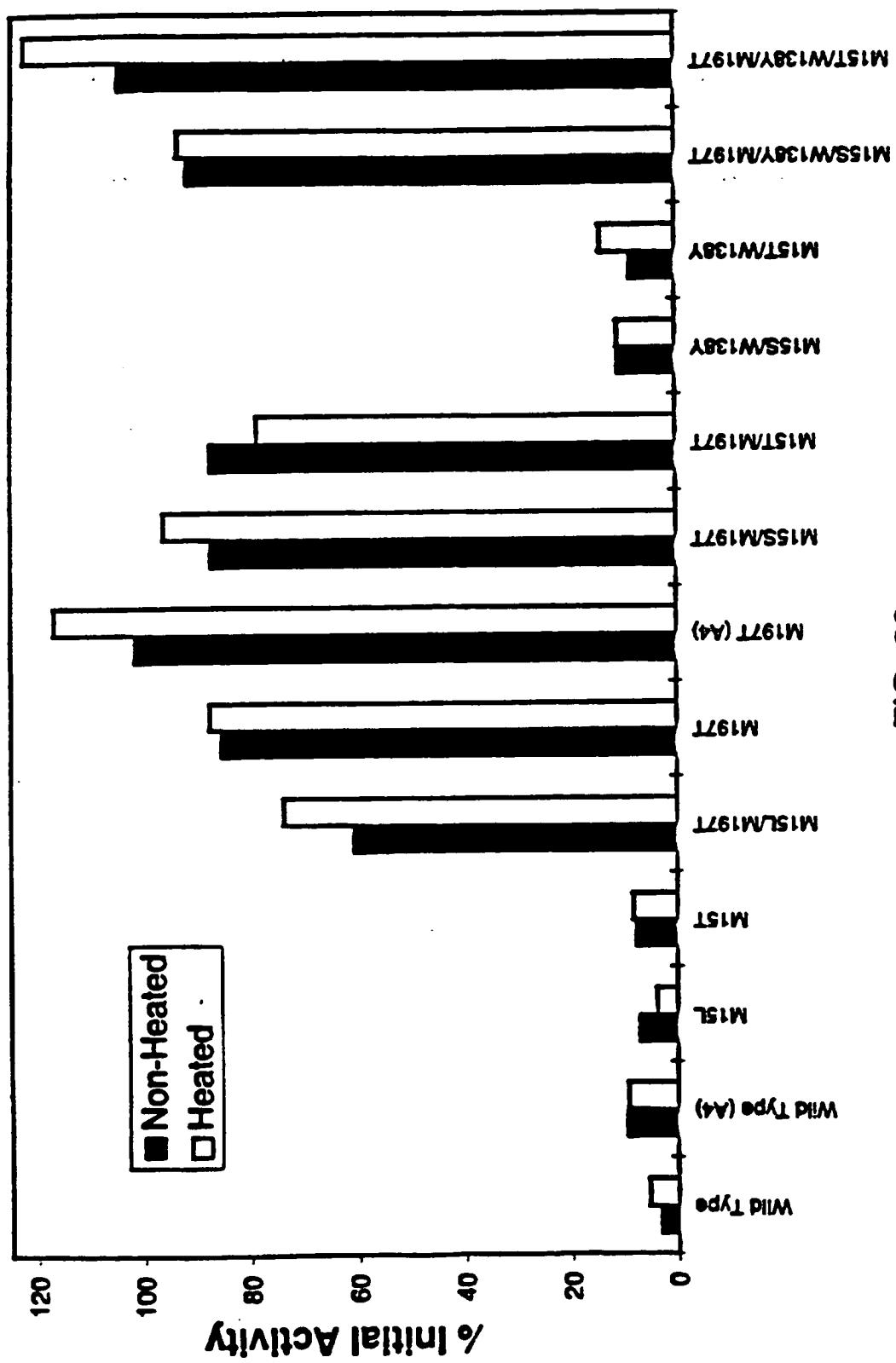


FIG. 20

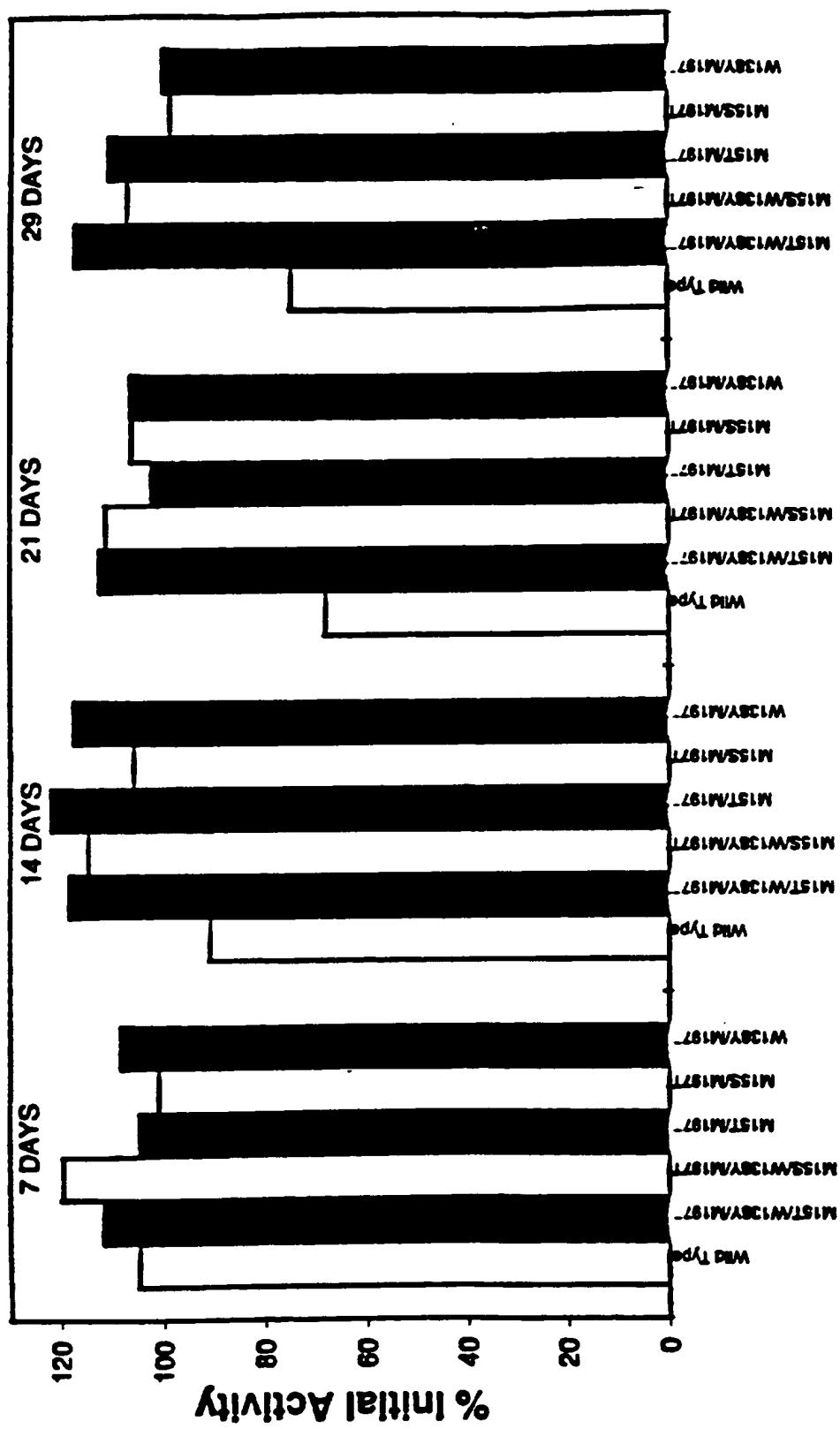


FIG. 21

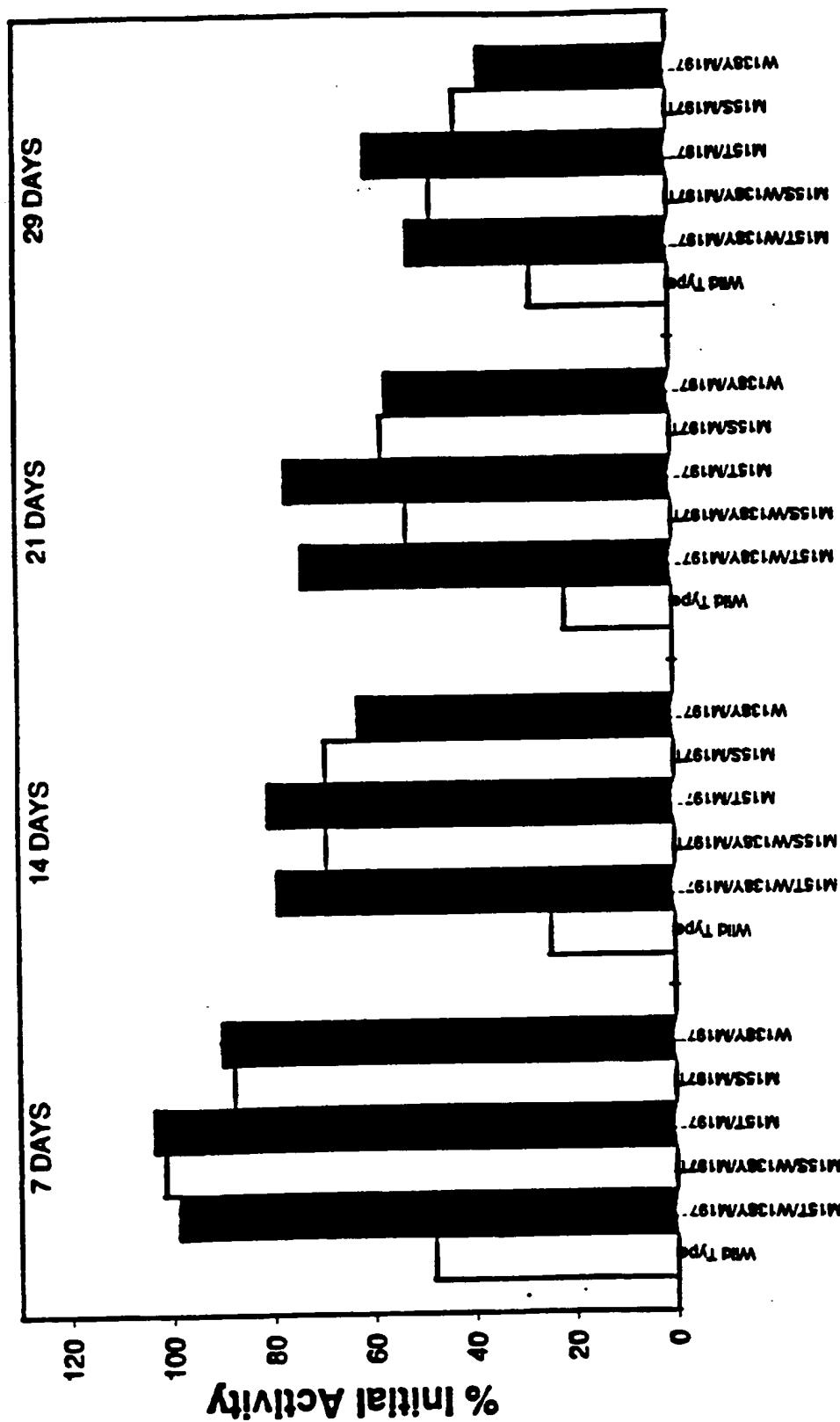


FIG. 22

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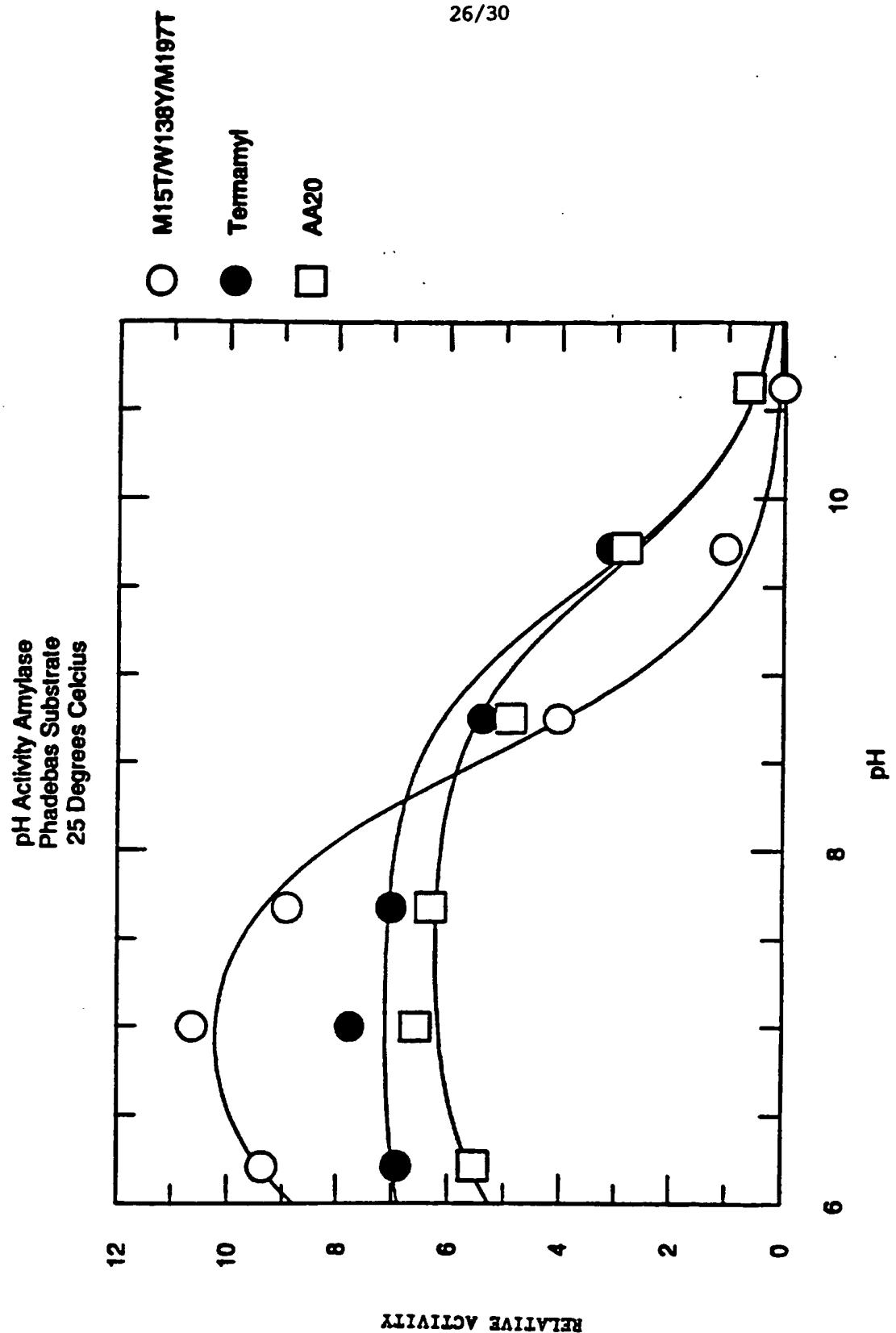
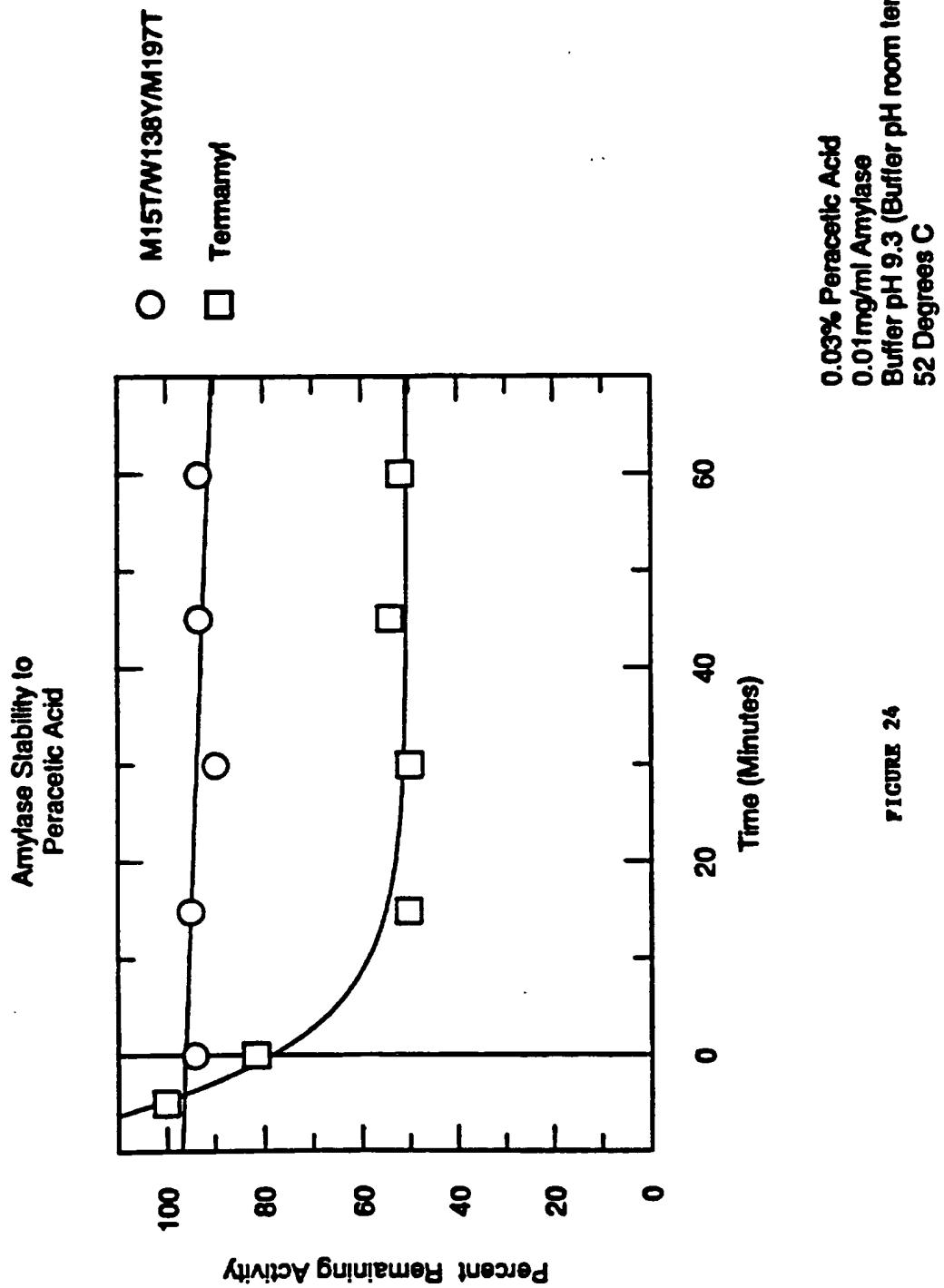


FIGURE 23



Wash Performance of M157/W138Y/M197T and TemamyI 6T in Liquid Detergent @40°C

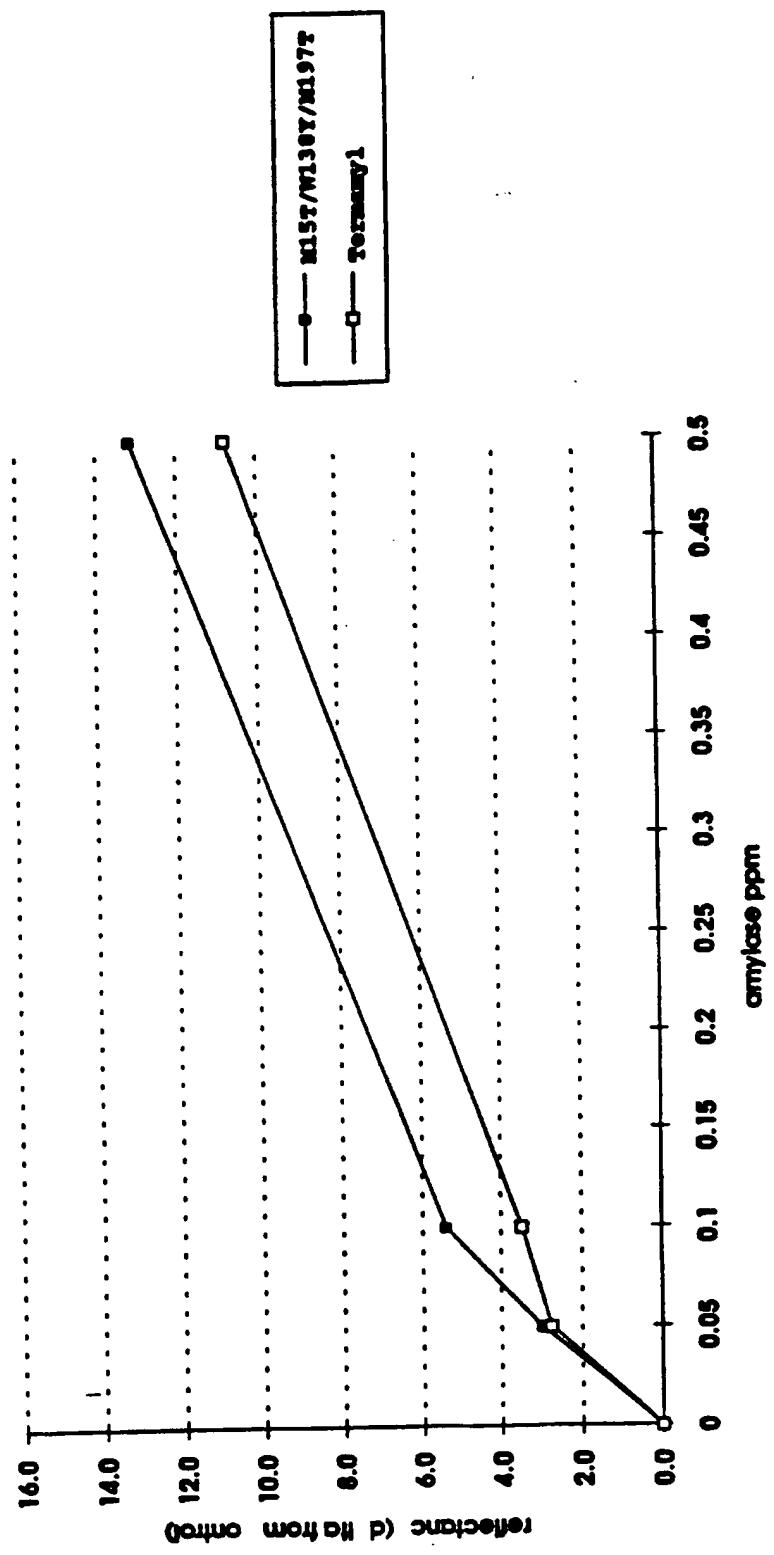


FIGURE 25

Wash Performance of M157/W138Y/M197T and Temamy 60T in Liquid Detergent @55°C

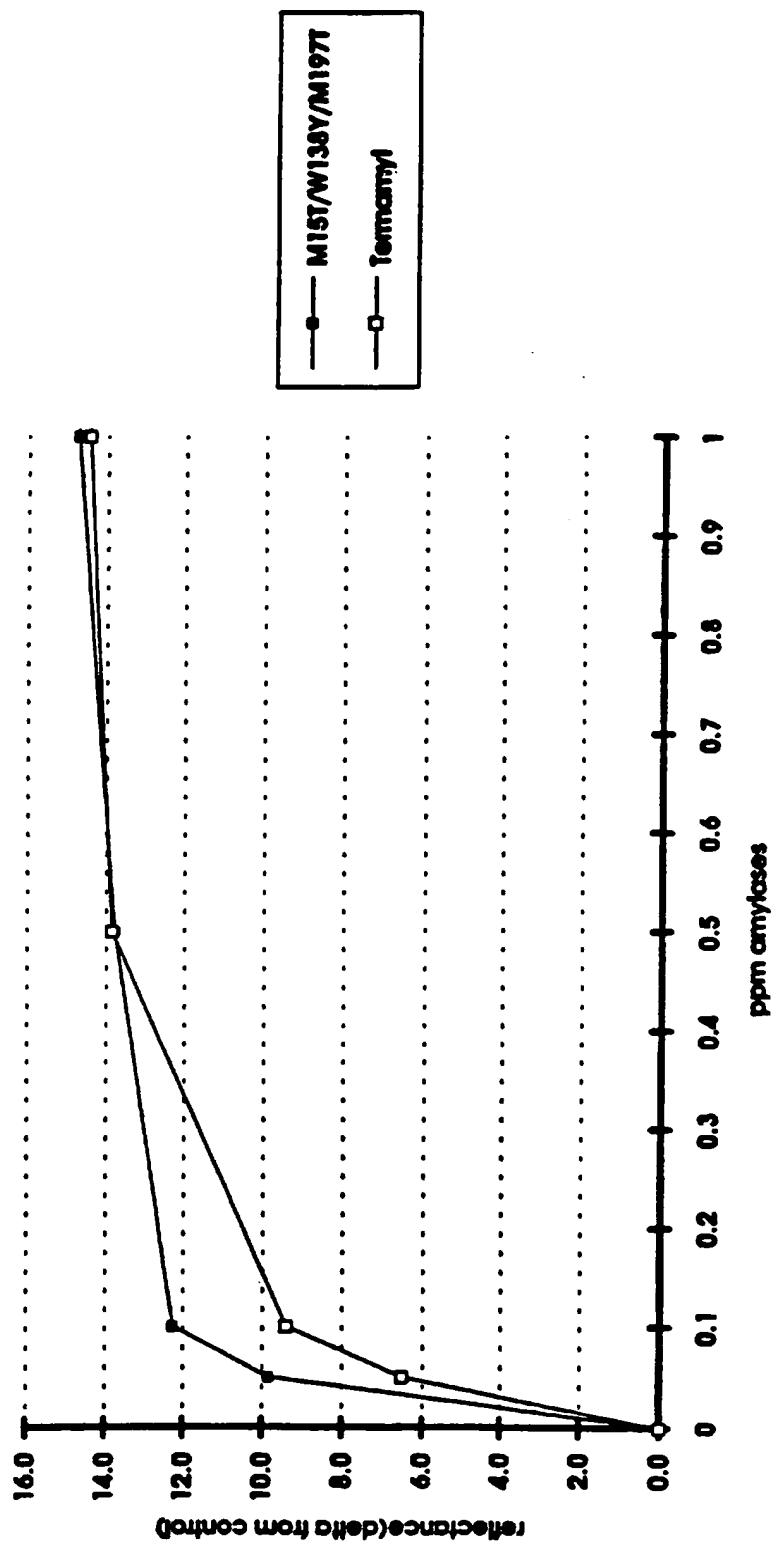


FIGURE 26

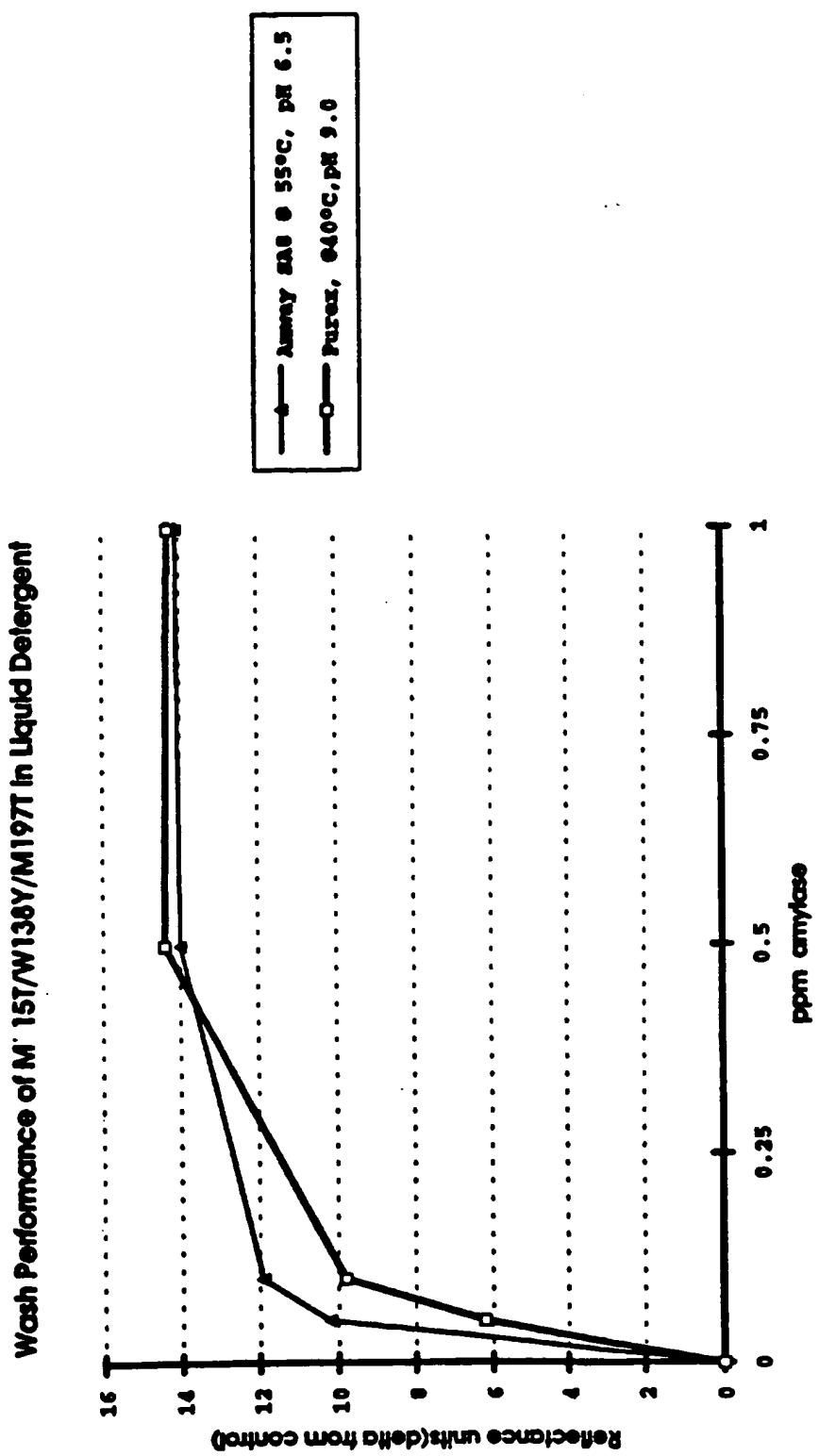


FIGURE 27

# INTERNATIONAL SEARCH REPORT

Int. Application No  
PCT/US 96/04029

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C11D3/386 C12N9/28

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C11D C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 18314 (GENENCOR INT) 18 August 1994 see claims 1-15,27-34 ---	1,3
X	WO,A,94 02597 (NOVONORDISK AS ) 3 February 1994 see page 5, line 6 - page 6, line 9; claims ---	1-3,7,9, 10
P,X	WO,A,96 05295 (GENENCOR INT) 22 February 1996 see the whole document ---	1-10
P,X	WO,A,95 10603 (NOVONORDISK AS) 20 April 1995 see page 36, line 5 - page 40, line 9; claims -----	1-3,7-10

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- \*A\* document member of the same patent family

1

Date of the actual completion of the international search

24 July 1996

Date of mailing of the international search report

29-07- 1996

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Grittern, A

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International Application No  
PCT/US 96/04029

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		EP-A-	0722490	24-07-96
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